Università degli Studi di Genova

PhD Thesis

CXCR4/CXCR7-CXCL11/CXCL12 AXIS PROMOTES MALIGNANT PHENOTYPE IN PUTATIVE STEM CELLS FROM HUMAN MENINGIOMAS

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Abstract

Meningioma is the most frequent primary tumor of the central nervous system. The greatest percentage of meningiomas is benign tumors (WHO grade I). However, although surgical and radiotherapy techniques have significantly improved over the years, some meningiomas, independently from the grading, are refractory to multimodality therapies, and recur and/or undergo malignant transformation, representing an unsolved therapeutic challenge. Therefore, beside histopathologic benign appearance, biologically aggressive meningiomas need to be molecularly characterized, to identify novel therapeutic targets.

In malignant tumors, recurrence is mainly ascribed to the presence of cancer stem cells (CSCs) which are expression of tumor cell heterogeneity, and sustain tumorigenesis, metastasization and drug resistance.

CSCs are characterized by stem cell marker expression, self-renewal, and ability to differentiate into tumor-specific cell types. Recently, CSCs and their functional role have been also studied in benign tumors, including meningioma. A range of genes and proteins have been proposed to identify meningioma stem-like cells, among them CD105, a transmembrane glycoprotein, involved in angiogenesis and in the progression of a variety of tumors. Stemness, as well as cancer cell aggressive behavior, is a cell property strictly linked to tumor microenvironment: reciprocal interactions between growth factors, cytokines and chemokines released by both CSCs and other cell types forming the niche, modulate each other to sustain tumor growth. Chemokine signaling, and the CXCL11/CXCL12-CXCR4/CXCR7 system in

particular, drives cell proliferation and migration in several solid tumors. On these premises, this study is focused on the isolation and characterization of stem-like cells from post-surgical samples of human meningiomas, delving deeply into the role of this subpopulation in meningioma aggressive behavior. Moreover, we analyzed the contribution of CXCR4-7 receptors in the regulation of their biological properties. Twenty-eight primary cell cultures have been obtained from 35 meningiomas, and maintained in stem cell-permissive culture conditions to enrich in CSCs. Putative meningioma stem cells rapidly grow, form meningospheres and express stem markers, such as Sox2, NANOG, CD133 and Oct-4. Conversely, CD105 was not differentially expressed between stem-like cells and their "non-stem" counterpart, cells grown in serum-containing medium. Moreover, stem-like cells displayed high migratory capacity and *in vitro* angiogenic activity, supporting their malignant phenotype. Meningioma stem-like cells displayed a distinct chemokine-receptor profile from "non-stem" cell population, and selectively respond to *in vitro* CXCL11 and CXCL12 stimulation enhancing proliferation, migration and vascular mimicry. Pharmacological inhibition of individual CXCR4 or CXCR7 significantly impaired CXCL12- and CXCL11-induced proliferation, chemotaxis and vessel-like structure formation, therefore suggesting that these activities are mediated by both receptors. We speculated that these receptors act as heterodimers, formed upon ligand activation and that the blockade of one of them results in a complete inhibition of biological effects.

Overall our results, collected from a large number of meningioma cell cultures derived from different patients, allow the identification of a tumor subpopulation endowed with common stem cell-like features, and suggest that both CXCR4 and

CXCR7 signaling sustains meningioma stem cell phenotype. Prospectively, the isolation and culture of stem-like cells directly from the meningioma tissues will allow to test new therapeutic compounds to block meningioma growth and invasiveness, in particular for those tumors showing an unpredictable aggressive behavior. In this context, we propose that the CXCR4-7 chemokinergic system might represent a relevant pharmacological target.

1. Introduction

Neoplasia is a term derived from the ancient Greek *νέος (neo)* "new" and *πλάσμα (plasma)* "formation, creation". Neoplastic cells are characterized by abnormal and transformed phenotype undergoing excessive proliferation. Neoplasms form uncontrolled and uncoordinated tissues, which continue to grow, depending on the host for their nutrition and blood supply.

In the common medical language, a neoplasm is more frequently called *tumor* (swelling) and, the study of tumors is called oncology (from *oncos*, “tumor,” and *logos*, “study of”). Tumors are basically divided into benign and malignant subtypes, based on their potential clinical behavior.

- Benign tumors: present non-aggressive gross characteristics, implying that they will remain localized and are susceptible to local surgical removal. Patients generally have a positive outcome.
- Malignant tumors: are able to rapidly grow, invade and destroy adjacent structures, and to spread to distant sites through the metastatic process. They are referred to as a *cancer,* from the Latin word for "crab", for their ability to adhere to any part, similar to a crab's behavior.

Cancer is a leading cause of death worldwide, and it has been recently reported that 9.6 million deaths are estimated to have occurred in 2018 (https://www.cancerresearchuk.org/health-professional/cancer-statistics/worldwide-cancer#heading-One). Understanding the cellular and molecular abnormalities in cancer cells and the mechanisms involved in carcinogenesis, could represent a revolution in cancer treatment. In recent years,

basic and clinical research has moved in this direction, making important progresses, as detailed in the next paragraphs.

1.1 BRAIN TUMORS

Tumors arisen within the central nervous system (CNS) affect both young and adult people. Brain tumors encompass a larger proportion of childhood cancer, accounting for as many of 20% of all pediatric tumors.

The annual incidence of CNS tumors ranges from 10 to 17 per 100,000 persons for intracranial tumors and 1 to 2 per 100,000 persons for intraspinal tumors; about half to three quarters of these are primary tumors, and the remaining are metastases from peripheral lesions. Statistical analysis shows that malignant CNS neoplasms are often aggressive, representing one of the main causes of tumor-related death.

It is important to stress that brain tumors cause many social and psychological burned, since beside health problems the quality life of patients is altered.

Since even low-grade lesions may infiltrate large brain regions, serious neurological deficits represent the main symptoms, and if tumor cells spread to distant sites, the lesion cannot be radically resectable leading to a poor prognosis. Moreover, the anatomic site of these neoplasms can influence the clinical outcome independently from the histological classification due to local invasion and neurological effects (for example, a benign meningioma may cause cardiorespiratory arrest by compression of the medulla).

The 2016 CNS World Health Organization (WHO) classification includes molecular parameters, in addition to histology, to define tumor entities representing an important revision of the 2007 CNS WHO classification [1], as reported in the following Table 1.

Table 1 : WHO Classification of tumors of central nervous system

Diffuse astrocytic and oligodendroglial tumours	
Diffuse astrocytoma, IDH-mutant	9400/3
Gemistocytic astrocytoma, IDH-mutant	9411/3
Diffuse astrocytoma, IDH-wildtype	*9400/3*
Diffuse astrocytoma, NOS	9400/3
Anaplastic astrocytoma, IDH-mutant	9401/3
Anaplastic astrocytoma, IDH-wildtype	*9401/3*
Anaplastic astrocytoma, NOS	9401/3
Glioblastoma, IDH-wildtype	9440/3
Giant cell glioblastoma	9441/3
Gliosarcoma	9442/3
Epithelioid glioblastoma	*9440/3*
Glioblastoma, IDH-mutant	9445/3*
Glioblastoma, NOS	9440/3
Diffuse midline glioma, H3 K27M–mutant	9385/3*
Oligodendroglioma, IDH-mutant and 1p/19q-codeleted	9450/3
Oligodendroglioma, NOS	9450/3
Anaplastic oligodendroglioma, IDH-mutant and 1p/19q-codeleted	9451/3
Anaplastic oligodendroglioma, NOS	*9451/3*
Oligoastrocytoma, NOS	*9382/3*
Anaplastic oligoastrocytoma, NOS	*9382/3*
Other astrocytic tumours	
Pilocytic astrocytoma	9421/1
Pilomyxoid astrocytoma	9425/3
Subependymal giant cell astrocytoma	9384/1
Pleomorphic xanthoastrocytoma	9424/3
Anaplastic pleomorphic xanthoastrocytoma	9424/3
Ependymal tumours	
Subependymoma	9383/1
Myxopapillary ependymoma	9394/1
Ependymoma	9391/3
Papillary ependymoma	9393/3
Clear cell ependymoma	9391/3
Tanycytic ependymoma	9391/3
Ependymoma, *RELA* fusion–positive	9396/3*
Anaplastic ependymoma	9392/3
Other gliomas	
Chordoid glioma of the third ventricle	9444/1
Angiocentric glioma	9431/1
Astroblastoma	9430/3
Choroid plexus tumours	
Choroid plexus papilloma	9390/0
Atypical choroid plexus papilloma	9390/1
Choroid plexus carcinoma	9390/3
Neuronal and mixed neuronal-glial tumours	
Dysembryoplastic neuroepithelial tumour	9413/0
Gangliocytoma	9492/0
Ganglioglioma	9505/1
Anaplastic ganglioglioma	9505/3
Dysplastic cerebellar gangliocytoma (Lhermitte–Duclos disease)	9493/0
Desmoplastic infantile astrocytoma and ganglioglioma	9412/1
Papillary glioneuronal tumour	9509/1
Rosette-forming glioneuronal tumour	9509/1
Diffuse leptomeningeal glioneuronal tumour	
Central neurocytoma	9506/1
Extraventricular neurocytoma	9506/1
Cerebellar liponeurocytoma	9506/1
Paraganglioma	8693/1
Tumours of the pineal region	
Pineocytoma	9361/1
Pineal parenchymal tumour of intermediate differentiation	9362/3
Pineoblastoma	9362/3
Papillary tumour of the pineal region	9395/3
Embryonal tumours	
Medulloblastomas, genetically defined	
Medulloblastoma, WNT-activated	9475/3*
Medulloblastoma, SHH-activated and *TP53*-mutant	9476/3*
Medulloblastoma, SHH-activated and *TP53*-wildtype	9471/3
Medulloblastoma, non-WNT/non-SHH	9477/3*
Medulloblastoma, group 3	
Medulloblastoma, group 4	
Medulloblastomas, histologically defined	
Medulloblastoma, classic	9470/3
Medulloblastoma, desmoplastic/nodular	9471/3
Medulloblastoma with extensive nodularity	9471/3
Medulloblastoma, large cell / anaplastic	9474/3
Medulloblastoma, NOS	9470/3
Embryonal tumour with multilayered rosettes, C19MC-altered	9478/3*
Embryonal tumour with multilayered rosettes, NOS	*9478/3*
Medulloepithelioma	9501/3
CNS neuroblastoma	9500/3
CNS ganglioneuroblastoma	9490/3
CNS embryonal tumour, NOS	9473/3
Atypical teratoid/rhabdoid tumour	9508/3
CNS embryonal tumour with rhabdoid features	*9508/3*
Tumours of the cranial and paraspinal nerves	
Schwannoma	9560/0
Cellular schwannoma	9560/0
Plexiform schwannoma	9560/0

Melanotic schwannoma	9560/1
Neurofibroma	9540/0
Atypical neurofibroma	9540/0
Plexiform neurofibroma	9550/0
Perineurioma	9571/0
Hybrid nerve sheath tumours	
Malignant peripheral nerve sheath tumour	9540/3
Epithelioid MPNST	9540/3
MPNST with perineurial differentiation	9540/3
Meningiomas	
Meningioma	9530/0
Meningothelial meningioma	9531/0
Fibrous meningioma	9532/0
Transitional meningioma	9537/0
Psammomatous meningioma	9533/0
Angiomatous meningioma	9534/0
Microcystic meningioma	9530/0
Secretory meningioma	9530/0
Lymphoplasmacyte-rich meningioma	9530/0
Metaplastic meningioma	9530/0
Chordoid meningioma	9538/1
Clear cell meningioma	9538/1
Atypical meningioma	9539/1
Papillary meningioma	9538/3
Rhabdoid meningioma	9538/3
Anaplastic (malignant) meningioma	9530/3
Mesenchymal, non-meningothelial tumours	
Solitary fibrous tumour / haemangiopericytoma**	
Grade 1	8815/0
Grade 2	8815/1
Grade 3	8815/3
Haemangioblastoma	9161/1
Haemangioma	9120/0
Epithelioid haemangioendothelioma	9133/3
Angiosarcoma	9120/3
Kaposi sarcoma	9140/3
Ewing sarcoma / PNET	9364/3
Lipoma	8850/0
Angiolipoma	8861/0
Hibernoma	8880/0
Liposarcoma	8850/3
Desmoid-type fibromatosis	8821/1
Myofibroblastoma	8825/0
Inflammatory myofibroblastic tumour	8825/1
Benign fibrous histiocytoma	8830/0
Fibrosarcoma	8810/3
Undifferentiated pleomorphic sarcoma / malignant fibrous histiocytoma	8802/3
Leiomyoma	8890/0
Leiomyosarcoma	8890/3
Rhabdomyoma	8900/0
Rhabdomyosarcoma	8900/3
Chondroma	9220/0
Chondrosarcoma	9220/3
Osteoma	9180/0
Osteochondroma	9210/0
Osteosarcoma	9180/3
Melanocytic tumours	
Meningeal melanocytosis	8728/0
Meningeal melanocytoma	8728/1
Meningeal melanoma	8720/3
Meningeal melanomatosis	8728/3
Lymphomas	
Diffuse large B-cell lymphoma of the CNS	9680/3
Immunodeficiency-associated CNS lymphomas	
AIDS-related diffuse large B-cell lymphoma	
EBV-positive diffuse large B-cell lymphoma, NOS	
Lymphomatoid granulomatosis	9766/1
Intravascular large B-cell lymphoma	9712/3
Low-grade B-cell lymphomas of the CNS	
T-cell and NK/T-cell lymphomas of the CNS	
Anaplastic large cell lymphoma, ALK-positive	9714/3
Anaplastic large cell lymphoma, ALK-negative	9702/3
MALT lymphoma of the dura	9699/3
Histiocytic tumours	
Langerhans cell histiocytosis	9751/3
Erdheim–Chester disease	9750/1
Rosai–Dorfman disease	
Juvenile xanthogranuloma	
Histiocytic sarcoma	9755/3
Germ cell tumours	
Germinoma	9064/3
Embryonal carcinoma	9070/3
Yolk sac tumour	9071/3
Choriocarcinoma	9100/3
Teratoma	9080/1
Mature teratoma	9080/0
Immature teratoma	9080/3
Teratoma with malignant transformation	9084/3
Mixed germ cell tumour	9085/3
Tumours of the sellar region	
Craniopharyngioma	9350/1
Adamantinomatous craniopharyngioma	9351/1
Papillary craniopharyngioma	9352/1
Granular cell tumour of the sellar region	9582/0
Pituicytoma	9432/1
Spindle cell oncocytoma	8290/0
Metastatic tumours	

The morphology codes are from the International Classification of Diseases for Oncology (ICD-O) [742A]. Behaviour is coded /0 for benign tumours; /1 for unspecified, borderline, or uncertain behaviour; /2 for carcinoma in situ and grade III intraepithelial neoplasia; and /3 for malignant tumours. The classification is modified from the previous WHO classification, taking into account changes in our understanding of these lesions.
*These new codes were approved by the IARC/WHO Committee for ICD-O. /tab/cs: Provisional tumour entities. **Grading according to the 2013 *WHO Classification of Tumours of Soft Tissue and Bone*.

1.2 MENINGIOMA

1.2.1 Pathological classification

PATHOLOGY

Meningioma is the most common primary intracranial tumor of arachnoid origin in the adult. In the past, several neoplasms currently classified as meningiomas were reported with different medical terms, depending on the area of origin of the tumor. In 1922, Harvey Cushing first used the term of “meningioma” to describe a set of brain and spinal cord tumors developed in proximity to meninges.

The meninges, from ancient Greek μῆνιγξ (meninx), lit. 'membrane' are the membranes that envelop brain and spinal cord and their primary function is to protect the central nervous system. The three mammalian meninges are: dura mater, arachnoid mater and pia mater. Dura mater, the external layer, presents fibrous and resistant characteristics and, close to the skull adheres to the periosteum forming a single structure, the endocranium. Arachnoid, the middle meninges, is structurally similar to a spiderweb (from the Greek word “arachnoid”: spider), and is wet by the cerebro-spinal fluid (CSF). The pia mater is the inner meninges, directly in contact with the neuraxis. As previously mentioned, CSF is located between arachnoid and pia mater, acting as cushion for the brain to prevent mechanical injuries, immunological protection and regulation of cerebral blood flow. Brain choroid plexuses continuously produce about 500 mL of CSF per day which is absorbed in the arachnoid or in “Pacchioni” granulations.

Meningiomas manly originate from cells of the arachnoid cap (meningothelial cells). These cells are located at the apical part of the Pacchioni bodies and are exposed to the venous vascular flow, often within the dural sinus. In fact, arachnoid cells, characterized by high metabolic activity, are involved in reabsorption of CSF. Meningiomas usually develop as extra-axial tumors, occupying space within the cranial or spinal canal, but mostly are not able to invade the nervous tissue that is however shifted and compressed by the growing mass.

CLASSIFICATION

Meningiomas are among the most common intracranial tumors in adults (about 37% of CNS tumors), showing high heterogeneity respect to histology, localization, clinical behavior and molecular features [2]. According to the 2016 WHO classification of tumors of the CNS [1], meningiomas are classified into three grades of malignancy showing an increased risk of recurrence. Despite the correlation between brain invasion and recurrence and mortality rates in WHO grade I meningiomas was described by Perry in 1997 [3], prior 2007 WHO classification considered invasion a staging rather than a grading feature, and opted to discuss brain invasion as a separate heading. In fact, classification and grading of meningiomas did not undergo substantial revisions, except for the introduction of brain invasion as a criterion for the diagnosis of atypical meningioma, WHO grade II. Moreover, the clinical value of such grading is limited, since it did not satisfactorily reflect tumor biology as well as the poor outcome and high recurrence rate (20%) observed in grade I meningiomas, or resistance to therapies and lethality observed in subgroups of patients, thus suggesting the need of molecular and genetic

characterization to identify prognostic markers to identify tumor subsets with divergent clinical behavior. WHO grade II and III tumors can arise de novo or develop from lower-grade meningiomas.

Approximately more than 80% of meningiomas are benign and classified as grade I; 4-15% are atypical (grade II) and 1-3% anaplastic (grade III) [3, 4], in which there are obvious malignant histological and cytological features such as increased cell proliferation and undifferentiated phenotype. Grade II include tumors displaying 4–19 mitotic figures/10 HPF or brain invasion, or at least three of the following aspects:

- increased cellularity;
- small cells with a higher nucleus/cytoplasm ratio;
- prominent nucleoli;
- growth with an uninterrupted appearance or a sheet of paper;
- spontaneous or geographical necrosis foci.

Grade III is assigned to lesions with≥20 mitotic figures/10 HPF or sarcomatous carcinomatous histology.

Table 2 Meningioma subtypes according to WHO grade (2016 criteria)

HISTOLOGICAL SUBTYPE	WHO grade (2016 criteria)
MENINGOTHELIAL	I
FIBROUS (fibroblastic)	I
TRANSITIONAL (mixed)	I
PSAMMOMATOUS	I
ANGIOMATOUS	I
MICROCYSTIC	I
SECRETORY	I
LYMPHOPLASMACYTE RICH	I
METAPLASTIC	I
ATYPICAL	II
CHORDOID	II
CLEAR CELL	II
PAPILLARY	III
RHABDOID	III
ANAPLASTIC (malignant)	III

HISTOLOGICAL SUBTYPES OF MENINGIOMA

The majority of meningiomas are slow-growing benign neoplasms identified as WHO grade I. As reported in the Table 2, meningiomas present a variety of histological subtypes, among which meningothelial, fibrous and transitional are the most frequent. In the meningothelial subtype, the lobules of tumor cells are surrounded by thin septa of collagen. From a cytological point of view cells seem arachnoid cap cells, but they show evident morphological alterations with oval or round nuclei with dispersed chromatin, smooth nuclear profiles and small indistinct nucleoli. Fibrous meningioma is characterized by elongated cells similar to normal fibroblasts, with the formation of extended bundles that intertwine with each

other. The collagen matrix can be present in different quantities. Finally, transitional meningioma has intermediate characteristics between above two subtypes, with a mixed lobular and fascicular conformation, often with spiral bodies.

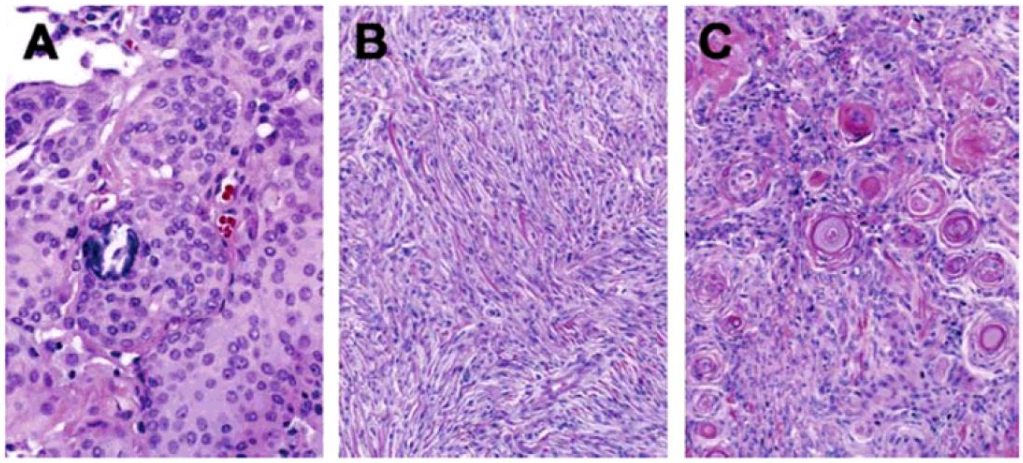

Figure 1 The most frequent histological subtypes of meningioma (grade I WHO) A) meningothelial; B) fibrous; C) transitional.

Others grade I meningioma subtypes are: psammomatous, angiomatous, secretory, microcystic, lymphoplasmacytic and metaplasmatic. Clinically, there are not differences between these benign histological types as far as growth behavior and prognosis.

Some histological subtypes are associated with a higher proliferative potential and recurrence rate and these correspond to the WHO II and III grades. Clear cell meningioma is a rare grade II subtype, presenting a benign histological appearance with an aggressive clinical outcome; WHO II chordoid meningioma resembles chordoma, a rare malignant bone tumor [5].

Anaplastic meningioma is characterized by histological and cytological aspects of evident malignancy, showing similar features to sarcoma, carcinoma or melanoma, and a higher mitotic index (20 or more mitosis per field) than atypical meningioma

[6]. Papillary and rhabdoid meningiomas are uncommon grade III subtypes, with an aggressive clinical course, high recurrence, metastatic diffusion, and mortality.

MOLECULAR ALTERATIONS

Recent studies show that the inclusion of new frequent molecular alterations in the diagnostic assessment might improve the identification of meningioma patients who need close surveillance and more-aggressive treatment [2]. Meningiomas display a high rate of copy-number alterations and karyotypic abnormalities. In particular, the most common cytogenetic alteration is loss of long arm of chromosome 22, which affects the neurofibromatosis 2 (NF2) gene, which encodes merlin, also known as schwannomin. Abnormalities in this gene result in the NF2 familial syndrome, which is characterized by the development of benign tumors of nervous system [7].

Most neurofibromatosis-associated meningiomas have mutations of NF2 gene or loss of chromosome 22 [8], however non-NF2 tumors are also present, commonly displaying alterations of phosphatidylinositol-3-kinase and Sonic hedgehog pathways, TRAF7, KLF4 or POLR2A genes [9]. In WHO grade II and III tumors, beside some specific alterations (SMARCE1, BAP1, chromatin regulatory genes ARID1A and ARID1B), the most frequent mutation involves NF2 gene [9].

Table 3 Recurrent mutations observed in meningiomas. Modified from Bi W.L et al. 2018

WHO Grade & Mutant Gene	Comment
I	
NF2	Associated w/ chromosome 22 loss
TRAF7	
AKT1	
SMO	
PIK3CA	
KLF4	
POLR2A	
SMARCB1	
II	
NF2	
SMARCB1	Germline & somatic
SMARCE1	Germline & somatic; associated w/ clear cell subtype
TERT	Associated w/ histological progression in grade
III	
NF2	
BAP1	Associated w/ rhabdoid subtype
TERT	Associated w/ histological progression in grade

BAM 22, a member of β-adaptin gene family, and MN1 genes, have been found frequently altered and, therefore, they are suspected of being involved in the initiation processes of meningioma development [10].

Other cytogenetic abnormalities, are especially described in atypical meningioma, include losses of 1p, 6q, 10q, 14q, 17p and 18q and acquisition of 1q, 9q, 12q, 15q, 17q and 20q [11, 12].

Some interesting studies have shown association between the loss of the long arm of chromosome 14 and male sex, and a greater risk of recurrence among patients with benign meningioma [13].

To date, the prognostic impact of all the above described mutations is still unknown.

LOCALIZATION

As other intracranial tumors, meningioma aggressiveness and malignancy do not depend only on their histological grade, but also on the location. In fact, some benign lesions in “critical” brain regions could limit surgical access and serious symptoms might result from the “mass-effect” in vital cerebral nuclei. Patients mass that invades the brain tend to have a poor outcome.

It is surgically difficult removing meningioma on the undersurface of the skull, in particular in regions adjacent to important brain structures and main vascular systems. Incomplete surgical resection and residual tumor cells are associated with a higher risk of tumor recurrence.

The most frequent localizations of intracranial meningiomas are:

- Falx and parasagittal (25%)
- Convexity (20%)
- Sphenoid wing (20%)
- Olfactory groove (10%)
- Suprasellar (10%)
- Posterior fossa (10%)
- Intraventricular (2%)
- Intraorbital (<2%)
- Spinal (<2%)

MORPHOLOGY

At macroscopic level meningiomas grow as well-defined tumors. They present nodular, round or oval shape, red-grayish color and, homogeneous and often compact solidity. Meningioma usually compresses the brain without infiltrating it. In spite of this, extension into the overlying bone and infiltration into the parenchyma may be present, not necessarily constituting malignant properties.

1.2.2 Epidemiology

AGE AND SEX

Meningiomas, with an estimated incidence of 7.86 cases per 100.000 people per year, primarily occur in elderly individuals, more frequently in the sixth and seventh decade, showing an increasing incidence over 65 years of age[14]. In adults, meningiomas preferentially affect women, with a female:-male ratio of 3.5:1 [15], which, in case of spinal lesions, rise to 10:1 [16].

On the other hand, atypical and anaplastic meningiomas are more frequent in men at younger age (median 57-year old). The different incidence could be related to the hormonal profile or genetic difference although, nowadays, no definitive evidence confirms this hypothesis [17].

In latest decades the incidence of meningioma is increasing, especially in elderly [18, 19]. This is certainly linked to a higher frequency of radiological investigations of the skull, to improved imaging techniques (computerized tomography and/or magnetic resonance imaging scan), and to increased population longevity [20]. In line with these assumptions, recent neuro-radiological registers report more new

cases of asymptomatic meningiomas casually detected in the elderly during analysis for different pathological conditions, but that, however, do not necessarily require surgery or hospitalization[21, 22].

Meningiomas are rare in childhood, representing 0.4-4.1% of all pediatric tumors, and equally distributed in both female and male [15]. Moreover, the incidence of meningiomas is increasing among adolescents and young adults, representing ~16% of all intracranial tumors in people 15-39 years of age [14].

1.2.3 Etiological and risk factors

GENETIC AND EPIGENETIC PREDISPOSITION

A genetic predisposition is observed in meningioma is observed in rare hereditary cancer syndromes, such as the most neurofibromatosis type 1 and 2, Turner's and Werner's syndrome [23, 24].

Neurofibromatosis type II syndrome, which is phenotypically characterized by the development of benign tumors of CNS, primarily schwannomas and meningiomas [7]. NF2-knockout mice develop spontaneous meningioma [25], endorsing NF2 as an initial oncogenic driver in both low- and high- grade meningioma tumorigenesis [26].

Besides, the losses of chromosome 1p and 14q are the second most frequent cytogenetic alterations observed in meningioma, and they affect 50% of grade II and almost grade III meningiomas [27, 28].

In meningioma initiation processes other genes are involved: BAM 22, a member of the β-adaptin family, and MN1, which is frequently altered in meningioma [29],

TRAF7 (*TNF receptor-associated factor 7*), AKT1 proto-oncogene, KLF4 (*Kruppel-like factor 4*), PIK3CA oncogene, and SMO (*Hedgehog pathway signaling member smoothened*) [30].

Patients with congenital and familial diseases, such as endocrine neoplasia type 1, Gorlin-, Cowden-, Gardner-, Turcot-, von Hippel-Lindau-, and Li-Fraumeni syndromes also have a higher incidence of meningioma.

HORMONE RECEPTORS

The highest incidence in women, the highest percentage of malignant meningiomas in men, the presence of progesterone (80%), estrogen (40%) and androgens receptors (39%) in these tumors, and the protective effects of pregnancy, suggest that sexual hormones play a role in meningioma development. In fact, epidemiological and biological data show a possible relationship between hormones and the risk of developing a meningioma. The preponderance of progesteron receptors (PRs) and the lack of estrogen receptors (ERs) in meningiomas are largely studied. The expression level of PRs in meningiomas determines a favorable clinical and biological outcome. Conversely, a reduction of PRs or the presence of ERs in meningiomas signals a higher presence of cytogenetic abnormalities, and an increasing aggressive behavior, progression, and recurrence [31].

Exogenous hormones with contraceptive or hormone-replacement therapy appear to have not a significant protective effect. Nevertheless, studies show long-term hormone-replacement therapy implication in tumorigenesis [32][20].

Nevertheless, currently the role of hormone receptors and hormonal therapy use in meningioma development is still controversial and not definitely proven [17, 33],

partly due to the limited number of case reported and epidemiologic data heterogeneity.

ENVIRONMENTAL FACTORS

Electromagnetic Radiation

In several epidemiological studies, exposure to ionizing radiation has been correlated with a higher incidence of meningioma. Treatment of *tinea capitis* of the scalp with low dose irradiation with 8 Gray (Gy), results in single or multiple meningiomas with an increased risk of 2.3% after a 35-year latency period [34]. Patients with gliomas, leukemias, lymphomas and/or brain metastases treated with radiotherapy, develop meningiomas after a slightly shorter median latency period (about 24 years). In general, meningioma induced by radiation exposure presents a more aggressive phenotype (about 50% are atypical). Among 80, 160 survivors to atomic explosions in Hiroshima and Nagasaki, 88 meningioma cases have been reported, corresponding to a risk of 6.48 times higher than the unexposed population [35].

Furthermore, exposure to low dose irradiation as dental x-rays appears responsible for an increased risk of meningioma [36-38]. In particular, dental X-rays performed in the past, when radiation exposure was greater than today, and performed frequently at a young age, appear to be associated with risk of intra-cranial meningioma [39].

Nowadays it is debated if chronic low exposures to mobile phone electromagnetic fields, classified as potential carcinogenic to humans by the International Agency for Research on Cancer, can be a potential risk factor for tumorigenesis. Studies report

conflicting data likely because the follow-up period relatively short, since emerging telecommunication techniques have become widely used only in the past 30 years, does not allow to provide consensus data [4, 20].

Chemical agent exposure

Chemical agents and compound, such as hair dyes, have been hypothesized as potential risk factors for meningioma onset, although to date no statistically significant data have been reported.

A harmful effect of lead has long been known as well as its connection to different brain tumors (in addition to those of stomach, lung and gallbladder), since this metal is able to cross the blood-brain barrier. Individuals exposed to lead constantly for professional reasons show a higher risk of developing meningioma than the unexposed population; in particular, this relationship involves subjects with ALAD2 allele, because G177C ALAD polymorphism influences the toxic kinetic of lead [40].

Head injury

Accident- or crash-related head injuries may lead to several brain, skull, and blood vessels damages, involving a massive flow of cytokines, histamine and bradykinins in extravascular space due to local alteration of the blood-brain barrier.

An analysis of approximately 3,000 people with head injury and 30,000 cases of control, the incidence of subsequent cranial tumors is not associated with the severity or location of the head damage [41]. Besides, the lack of etiological role of brain traumas in the onset of meningioma was also suggested by the observation than meningiomas are more common in women while cranial trauma are 2-3 times more frequent in men.

PREDICTING OUTCOME

Most meningiomas have a good long-term outcome, however, the prognosis is variable among individuals on the basis of a complex background of tumor recurrence. The 5-years survival of benign meningiomas is about 70%, it declines in grade II and III (65%) and in older patients. Beside complete surgical resection the 5-year recurrence rate of grade I meningiomas is about 20%, and 10-year recurrence is 10% in grade I, 30–40% in grade II and 50–90% in grade III. WHO grade III meningiomas, have a poor median overall survival of 1.5 years [42].

High histological grade, papillary morphology, uncompleted surgical resection, age, male gender, low performance status, involvement of optical nerve, and a high mitotic index are prognostic factors associated with a higher rate of relapse [43, 44].

The age of the patient influences the outcome. Generally, younger patients undergoing surgery recover better than older patients, in which more complications can arise. Tumor location and its surgical accessibility is a critical factor determining factor for patient survival, since radical resection cannot be always possible and, therefore, patients undergo partial resection allowing residual cells to re-grow and cause relapse [45].

A high mitotic index and early recurrence are often associated with meningiomas characterized by the absence of progestin receptors [46].In addition, chromosomal abnormalities and loss of heterozygosis are associated with lower survival and one higher relapse rate [13, 47].

PROLIFERATION INDEX

Proliferative potential is measured with different proliferation markers, usually by immunohistochemistry with the monoclonal antibody MIB-1, which targets the antigen Ki-67. Ki-67 is a nuclear protein present only during active phases of the cell cycle (phases G1, S, G2 and M). Ki-67 index is calculated as a percentage of positive nuclei of tumor cells in relation to the total nuclei number examined in multiple microscopic fields [16]. Ki-67 expression is significantly related to a higher histopathological grade and an increased risk of recurrence in meningiomas [20, 48]. However, although Ki-67 index may provide valuable additional prognostic information, it is not included in the classification criteria, in virtue of a high inter-individual variability [49, 50].

Proliferative or tumorigenic potential of meningioma are also dependent on the levels of vascularization: a higher amount of vascular endothelial growth factor (VEGF) expression is significantly associated with meningioma vascularization and proliferation [51].

1.2.4 Diagnosis

Symptoms

Despite their slow growth, intracranial meningiomas could remain completely asymptomatic for a long time. At the observation, many patients present a meningioma with a great bulk and poor or no neurological symptomatology.

In some cases, due to the low correlation between tumor volume and clinical symptoms, the clinical history could be lasting for decades. Symptoms appear when

tumor bulk becomes so big to compress the intracranial structures. Independently from the tumor location, a slowly progressive intracranial hypertension syndrome invariantly occurs. Common symptoms are: headache, vertigo, nausea or vomiting and, papillary stasis. Less frequently, patients may develop transtentorial herniation and, cerebellar tonsils with loss of consciousness, uncontrolled eye movement, loss of respiratory capabilities, trouble talking, and epileptic seizures. A specific symptomatology, instead, is related to the specific meningioma location. The most frequent sites of tumor development are parasagittal and falx areas, followed by convexity and sphenoid wing.

Compression symptoms are: focal seizures, edema and irritation of the cortex; these are followed by neurological deficits and signs of intracranial nerve involvement, especially in meningiomas grown in the cranial base.

Imaging

Nowadays there are no indications for a specific screening for meningioma detection. Patients with recent epileptic seizures or focal neurological signs, with suspected presence of an intracranial tumor mass, are examined by brain Magnetic Resonance Imaging (MRI). The majority of focal extra-axial masses are meningioma [52]. In many cases, standard radiography of skull, currently replaced by Computed Tomography (CT), is nevertheless able to highlight:

- Meningeal vascular grooves dilatation and hypertrophy of the meningeal vessels due to increased vascularization;
- Bone erosions or hyperostoses and, in some cases, an osteoma;

- Psammomatous tumor calcifications, although rarely, may define meningioma contour [53].

Angiography facilitates a precise visualization of tumor vascularization, including origin and location of tumor vessels. The typical pattern is the early appearance of contrast medium in the arterial phase and, its persistence in the venous phase. Angiography also helps in identifying occlusions of cerebral sinuses, mainly present in parasagittal and falx meningiomas.

At CT and RM scans, meningiomas appear sessile, pedunculated or, more rarely, "carpet" at cranial base level and, due to high vascularization, they occur as isodense or hyperdense masses of dural surface with a mottled characteristic structure [53-55]. This aspect can be evaluated by digital bidimensional-angio RM for vessels visualization instead of trans catheter angiography [55, 56].

By perfused MRI, peritumoral edema and vessels distribution have been studied in the interested brain area. Malignant histological features, large size, and basal localization are associated with a peritumoral edema, and correlated with a decreased blood flow in cerebral adjacent regions [57].

By CT, psammomatous calcifications, which are not identified by MRI, are clearly evident.

In radiological uncertain cases, for a "better" classification another diagnostic technique is MRI associated to alanine, high levels of choline and glutamine and low concentrations of creatinine, N-acetyl-aspartate and lipids spectroscopy [52, 58].

In addition, before surgery, it is possible to use perfusion RMI, the arterial spin labeling technique, or the kinetic PET (positron emission tomography) providing information on the vascular supply of meningiomas [59].

1.2.5 Meningioma treatment

Surgery

Surgery represents the first choice treatment for symptomatic meningiomas and, generally, it is able to solve the majority of cases, relieving tumor mass symptoms and allowing histological diagnosis [52]. Conservative treatment or only observation by clinical and MRI follow-up, is indicated in small and asymptomatic meningiomas, especially in elderly patients [53]. All modern imaging techniques combined with neurosurgery and micro-neurosurgery make possible the successful removal of tumors previously considered non-operable, for difficulties in the surgical access, or for their strict connection with vital brain structures.

The most reliable predictive factor of meningioma recurrence is the extent of surgical resection. For grade I meningiomas, Simpson grading scale plays an important role in guiding their treatment since 1957 [60]. Quality of the surgical resection is evaluated on the estimate extension of the resection through a classification still used and adapted according to the latest imaging techniques [52].

The extent of surgical resection, graded by the Simpson five-level system, is divided in:

- grade I: total resection of tumor;
- grade II: tumor is macroscopically totally resected and dural attachments coagulated;
- grade III: total resection without coagulation of dural attachments or incomplete resection;
- grade IV: partial resection with visible macroscopic tumor residue;

- grade V: biopsy.

Current European Association of Neuro-Oncology (EANO, 2016) guidelines [61] for post-surgical treatment and follow up of meningioma patients are based on the Simpson grading and tumor grade (Table 4).

Table 4 EANO guidelines for meningioma treatment with respect to WHO grade and extent of resections. Adapted from Goldbrunner et al., 2016. WHO, World Health Organization [9]

WHO grade	extent of resection	adjuvant treatment	follow-up
WHO grade I	Simpson grade I-III	Observation	3 months, annually for 5 yrs, then every 2 yrs
	Simpson grade IV-V	Radiosurgery	
WHO grade II	Simpson grade I-III	Observation or fractionated radiotherapy	3 months, biannually for 5 yrs then annually
	Simpson grade IV-V	Fractionated radiotherapy	
WHO grade III	all Simpson grades	Fractionated radiotherapy (plus experimental therapy)	every 3-6 months

Recurrence rates for spinal meningiomas are smaller than for the intracranial ones [62].

Endovascular embolization

Embolization results in tumor necrosis and it can be used preoperatively to separate tumor from highly vascularized tissues, as bone, *dura mater* and cerebral parenchyma. Furthermore, embolization reduces morbidity and mortality associated to intra-surgery bleeding.

Arteries that spray a meningioma, mainly originate from the external carotid artery. An endovascular approach allows to reach and to remove tumor capillaries preserve in the arteries of healthy tissues. In this way, it is possible to avoid compromising

wound healing in the postoperative period [63, 64]. Embolization can also be taken into account as a palliative procedure [65].

Then, tumors shrink and become softer and easier to resect. Nevertheless, the overall risk of embolization is 5-6%. Accidental embolization of a particular vessel could determinate serious complications into the intracranial circulation or intratumoral hemorrhage or a cranial nerve injury. The common embolic agent used is polyvinyl alcohol (PVA). Large PVA particles have lower risk of penetrating adjacent tissues and causing stroke or cranial nerve palsy. Conversely, smaller particles get deeply into the tumor but may also migrate into normal tissue [66]. In addition to PVA particles, the Onyx liquid embolic agent can be used. Seven to 9 days is the optimal timing for surgical procedure after embolization [67]; after that, there is substantial potential for the perfusion recovery of embolized vessels.

Radiation therapy

Irradiation represents first-line treatment, an adjuvant treatment, or a second-line treatment for recurrent meningiomas. Radiotherapy is the first-line treatment for skull-base meningiomas involving structures such as the cavernous sinus and the optic nerve, and as the only therapy when surgical approach is unfeasible.

Meningioma cells are relatively radio-resistant but still, radiotherapy is the most effective adjuvant therapy available. Generally, fractionated radiation is used in a 5 to 6 weeks treatment time, for partially resected benign meningiomas to sustain surgery and, usually total 50 to 60 Gy doses are needed to control the tumor growth for prolonged times. In atypical or malignant meningiomas and, in patients undergoing only partial tumor removal, fractionated radiotherapy is particularly

indicated [20, 52]. For these aggressive tumors, higher total doses are required fractionated radiotherapy (>54 Gy in 1.8 to 2.0 Gy fractions) [68]. In spite of the advantage offered by modern radiotherapy, neurotoxicity due to this treatment can potentially affect the quality of life of patients; in particular radiotherapy effects on pituitary gland, on cranial nerves and cognitive functions have not been completely defined yet [69].

More sophisticated radiotherapy techniques allow a major disease control in about 80 - 90% of cases. Generally, a total dose of 54 Gy (45-70 Gy) results effective and delayed further recurrence [70, 71]. Stereotactic radiation therapy (SRT) is a treatment that, through a high single dose, hit a small volume of the brain (about less than 3 cm) without affecting surrounding tissues. For these reasons, radiosurgery has recently become the first choice for well circumscribed, intracranial meningiomas and large inoperable tumors. CTT and MRI imaging techniques are used to better define the target volume to be hit by radiotherapy.

In one or more therapy sessions, through a linear accelerator or, in a single fraction therapy using a gamma-knife, SRT permits to attack tumors close to critical area such as cranial nerves, making resectable tumors that can only partially be removed by surgery [72]. Stereotactic radiosurgery (SRS) with the gamma knife technique, is currently widely used to treat intracranial meningiomas of size<3–4cm. SRS determines common side effects such as cranial nerve defects and peritumoral edema and more rarely necrosis, peritumoral cyst formation, carotid artery stenosis, and hypothalamic dysfunction [73].

MEDICAL TREATMENT FOR RECURRENT MENINGIOMAS

Chemotherapy

Systemic therapies are used after surgery and radiotherapy failure, however effective drugs for meningiomas are presently lacking [74].

Therefore, chemotherapy is rarely used in the treatment of meningiomas, as adjuvant treatment after surgery and radiotherapy [28]. Short- and, mainly, long-term effects such as lymphomas and leukemias, pulmonary fibrosis, renal insufficiency and neurotoxicity justify the chemotherapeutic approach only in a minority of cases. Classical cytotoxic drugs (temozolomide, irinotecan, doxorubicin, ifosfamide) did not improve patients' outcome [74]. Furthermore, since benign meningiomas have a slow growth rate, it is difficult to evaluate the effect of chemotherapy.

Hydroxyurea (HU), a ribonucleotide reductase inhibitor, used in patients who underwent complete tumor resection at 20 mg/kg/day, was able to prevent malignant meningioma recurrence for 24 months [75, 76]. Subsequently, numerous clinical reports on patients with recurrent or progressive meningioma revealed efficacy of HU antitumor treatment [77].

In a study, after treatment with HU, 18 out of 20 patients exhibited a stabilization of the pathology and a partial response in 2 cases, for an observational period of 80 weeks. Hematological side effects have often arisen, mainly characterized by neutropenia [78].

Moreover, in 12 benign meningioma cases, the disease was stabilized and two of patients showed clinical improvement. One patient manifested a minor partial

response that was noted after 3 years of treatment on neuroimaging and clinical evaluations. Chemotherapy was generally well tolerated, although in some patients a minor haematological toxicity was reported, and only one patient interrupted therapy due to moderate myelo-suppression [79]. Another clinical trial reports benefit in the majority of patients treated with HU: 11 of 15 patients obtained a stabilization of the disease for a median duration of 11 months [80]. The disease stabilizing effects of HU have not been confirmed by other studies. Hydroxyurea only has showed a stabilizing activity in some series [70–72], but this has not been consistently confirmed [81, 82].

Several small phases II studies using a combination treatment of HU with other chemotherapeutic agents as imanitib did not reported effects on patients' survival [83].

Verapamil, a calcium channel antagonist, known to augment the effects of chemotherapy drugs through the inhibition of the extruding pump P-glycoprotein, combined with HU, reduce vascularization, size and cell proliferation with an increase of apoptosis rate [84].

The post-radiation co-treatment with cyclophosphamide, adriamycin and vincristine (CAV), caused a very modest improvement in the median survival of 5.3 years respect to a median survival of 2.7 years in a group of patients who received subcutaneous octreotide three times per day; nevertheless co-treatment CAV was associated with high toxicity [28]. In particular, different chemotherapy-related complications occurred as transient alopecia, anemia, thrombocytopenia, and neutropenic fever; however, no bladder or cardiac toxicity appeared and no patients died as results of chemotherapy-related toxicity [85].

Temozolomide (TMZ), a chemotherapeutic agent used to treat glioblastoma multiforme (GBM) and astrocytomas, is not effective in recurrent meningiomas [86].

Irinotecan, an antitumor drug especially used to treat colon and small cell lung cancers, showed anti-proliferative activity both *in vitro* in meningioma cell cultures and *in vivo* in animal studies, but no clinical efficacy was observed in a phase II clinical trial [87].

Targeted Therapy

Nowadays, little is known about the signal transduction pathways, the growth factors and their receptors involved in meningioma proliferation [88, 89].

Many studies, to evaluate possible novel molecular target driving cell growth, proliferation and angiogenesis, are ongoing. Meningiomas present aberrant expression of signaling molecules and deregulated cell signaling pathways as a driver of neoplastic transformation [28, 90, 91]. For example, Hedgehog (Hh) signaling pathway, crucial for embryogenesis and cellular growth, is significantly activated in some meningiomas [92]. Serine/threonine protein kinase AKT pathway regulates several cellular processes as crucial tumor proliferation and survival, tissue invasion, and resistance to chemotherapy. AKT, highly expressed in skull base meningioma, has been reported [93].

Hormonal Therapy

Women have a higher incidence of developing meningioma, especially after puberty and during their most fertile years. Moreover, the higher incidence in meningioma

reported among breast cancer patients support the potential role of hormones in both diseases and drugs used for breast cancer treatment have been used for hormone manipulation in meningioma.

Tamoxifen, an oral anti-tumor drug largely used in breast cancer patients, belongs to the selective estrogen receptor modulators (SERMs) family, and inhibits the estrogen effects by deacetylating histones and blocking DNA binding to estrogen-receptor. However, in meningioma patients tamoxifen did not significant response in term of tumor growth inhibition [94, 95]. The PR inhibitor mifepristone (RU486), a potent contraceptive designed in the 80's and predominantly used to induce early pregnancy interruption, acts at lower concentration than progestogens by inhibiting the transcriptional activity of the progesterone receptor [96].

The use of progesterone antagonists as palliative treatment for meningiomas is controversial. In few small trials, positive results were obtained [97, 98]. A prospective study by SWOG (Southwest Oncology Group) on 193 patients whose state of PR was unknown in 138 out of 193 patients, 80 patients were treated with mifepristone at a dose of 200 mg and 80 with placebo, for a median period of 10 months. Grade IV toxic and side effects were detected in 6 mifepristone-treated arm patients and in a placebo-treated arm patient. Grade III toxicity has been described in 30 mifepristone-treated patients and in 24 placebo-treated patients. The toxic effects were mainly constituted by hypostenia (72% versus 54%), headache (44% versus 41%), and flushing (38% versus 26%). A large number of female patients also presented endometrial hyperplasia [99].

Somatostatin receptors are also expressed in the majority of meningiomas [100]. *In vitro*, somatostatin inhibits meningioma proliferation. Preliminary study of 16

patients has shown a partial response to somatostatin treatment of one third of patients. In a phase II trial, subcutaneous treatment with octreotide, a somatostatin receptor agonist, resulted in 17 weeks PFS and a median survival time of 2.7 years in 11 patients with recurrent meningioma compared to previous trials that reported a median survival time of well under 1 year [101] [102]. Octreotide is also under study in combination with everolimus, a drug targeting the mTOR-pathway whose activity in meningioma cells has been proven *in vitro* [103], in recurrent tumors.

Anti-Angiogenic treatment

Blood vessels play an essential role in tumor nutrition. Meningiomas are characterized by high vascularization and, therefore, blood vessel formation represents a potential therapeutic target. For example, anti-angiogenic drug fumigillin analogue (TNP-470) inhibited the growth of benign and malignant meningioma in xenografts animal models [104].

Vascular endothelial growth factor (VEGF) is the most important molecule involved in tumor vascularization and anti-VEGF drugs are used in different cancer types [105, 106]. VEGF and VEGF-receptor expression is directly proportional to meningioma grading. In fact, atypical and malignant meningiomas express 2- and 10- fold higher levels of VEGF, respectively, than benign meningiomas [107]. The anti-VEGF antibody bevacizumab has been largely tested in clinical trials representing a well-tolerated treatment. Partial regressions have been reported in anaplastic meningioma, in which patient received bevacizumab intravenously every two weeks for 15 months after non-curative surgeries [108] and, in a NF2-deficient meningiomas [109]. A group of 15 bevacizumab-treated patients with atypical or

malignant meningiomas, reported a median PFS of 26 weeks, and PFS6 rate was 43.8% compared to a group of 10 imanitib-treated patients, in which the median PSF of 8 weeks, and PSF-6 rate of 0%, and to a group of 17 patients treated with the anti-somatostatin receptor agonist Pasireotide, in which the median PFS was of 16 weeks, and a PFS-6 rate was 20% [110] and a decreased tumor blood volume. Another retrospective study conducted on 15 patients with recurrent atypical and malignant meningioma and treated with bevacizumab, reports a PFS6 of 86% respect to a PFS6 of 10% observed in patients treated with the ribonucleotide reductase inhibitor (HU) [111]. Although well tolerated, different side effects such as CNS hemorrhage and/or intestinal perforation occurred due to bevacizumab treatment [111].

A phase II clinical trial on 36 patients with grade II and III meningioma was also performed using the VEGFR and PDGFR inhibitor sunitinib. Unluckily, 60% of patients suffered from grade III toxicities, 32% of them needed dose reduction, and 22% of patients had to interrupt the treatment [112]. Twenty-five patients with benign, atypical and malignant meningioma were also treated with four cycles of vatalanib (PTK787), another VEGFR and PDGFR inhibitor. Vatalanib proved to be safer and less toxic than sunitinib prolonging PFS; in patients with recurrent Grade II and III meningioma treated with sunitinib the median PFS was of 4.6 months, instead patients treated with vatalanib the median PFS was of 7.6 months, [113]. These promising results should be confirmed in larger prospective phase III randomized clinical trials [28].

Interferons

Several evidence supports that interferon-alpha (IFNα) promotes the inhibition of meningioma cell proliferation *in vitro* [114-116]. Only small studies, however, demonstrated the IFNα effect on the progression of recurrent and unresectable meningiomas. Interferon-alpha helps stabilizing or diminishing recurrent meningiomas. Five of six patients treated with IFNα for five days a weeks showed stable disease up to 14 months and, only one had minor reduction of the tumor [117]. Another report reveals a stable disease up to 8 years in 9 out of 12 patients [115]. Recently, in a larger study, involving 35 patients with grade I recurrent meningioma, 10 patients had some toxic effects and, consequently, a reduction of the drug dose; 25 patients had a stable disease with median time to tumor progression of 7 months. On the other hand, 9 patients progressed with a median survival time about of 8 months [118]. The outcome of INF-α treated patients is quite various and unpromising [107, 119, 120].

Meningioma animal models, such as transgenic mice in which NF2 and CDKN2ab are inactivated, might be helpful to further evaluate the therapeutic efficacy of interferons [121].

Oncolytic Virus

An emerging medical treatment for different tumors is the use of oncolytic virus (OV) to determine an anti-tumor cellular immune response. This type of virus is able to kill selectively tumor cells. Actually, different OVs, including adeno and herpes simplex (G47D) viruses, have been investigated in preclinical meningioma models.

G47D is also in clinical trials for recurrent glioblastoma and in schwannoma, and preliminary results suggested its safety and encouraging efficacy [122, 123].

Immunotherapy

The most recent medical strategy in oncology is represented by immunotherapy, based on the inhibition of immune checkpoint modulatory molecules, whose expression has been detected also in PD-L1-cell infiltrating aggressive meningiomas [124]. Clinical trials are ongoing with the anti-PD-1 agents nivolumab agents nivolumab, pembrolizumab (NCT03173950) and avelumab (in combination with radiotherapy) in relapsed high-grade meningiomas.

LONG-TERM TOXICITY OF THERAPEUTIC APPROACHES

Cognitive and neurological deficits may have a significant impact on patients with brain tumors, independently from the histological subtype and grade of neoplasia. In particular areas, surgery causes cognitive deficits: the major problems are loss of memory, apathy, difficult in concentration and personality changes. Radiotherapy effects on cognitive functions are still unclear likely due to the difficulty to determine if deficits were induced by the tumor or the treatment [125, 126]. A recent study in patients with low-grade progression-free glioma did not confirm correlation between radiotherapy and cognitive deficit [127]. Only relationships between cognitive deficits and antiepileptic drugs have been identified. Epileptic seizures have a significant impact on the quality of life despite an effective control of the disease. New antiepileptic drugs with fewer side effects can be useful especially in patients under multi-chemotherapeutic treatment.

Radiotherapy can damage cranial nerves or induce endocrine dysfunction, also when the treated neoplasia is distant from the hypothalamic pituitary region [128]. Therapies also increase the risk of mortality. Chemotherapy induces late side effects, such as lymphomas, leukemia or solid tumors, pulmonary fibrosis, kidney failure, infertility and neurotoxicity. Also radiotherapy, at doses over 54 Gy, has a risk of mortality about of 2.5% at 2 years. In addition, at high and widespread doses, more than 5% of patients may present radio-necrosis, causing blindness if adjacent to the optic chiasm.

FUTURE PERSPECTIVE

The high frequency of recurrence and, the absence of an effective pharmacological therapy, for both benign (grade I) and malignant (grade II and III) tumors, are stimulating challenge for clinical management of these tumors. Moreover, both ongoing basic and preclinical research, foster new knowledge on these tumors. An interaction between clinical, epidemiological, pharmacological, genetic and biological research has recently allowed a therapeutic improvement constantly evolving; nevertheless, the persistent problem requires further insight.

1.3 STEM CELLS

Differentiated tissues are composed by heterogeneous cell subpopulations, and are constantly renewed through the differentiation of stem cells [129]. A balance between the replication, self-renewal and differentiation of stem cells and the death of mature and differentiated cells is necessary to maintain the homeostatic

equilibrium.

Two important features characterize stem cells:

- *self-renewal*: to maintain a relatively constant number of functional population of precursors for long periods of time by *asymmetric replication*: the ability to produce two types of daughter cells. One cell begins the differentiation pathway, giving rise to the progenitors and mature cells composing different organs, while the other cell retains its stemness preserving its self-renewal ability.
- differentiation into multiple lineages on more mature cells.

Moreover, stem cells display higher resistance to cytotoxic agents, included drugs, than differentiated cells, due to their high ability to repair DNA and the high activity of membrane transporters.

Normal stem cells can be identified according to the origin and the differentiation potential, but they can be essentially and divided into two types:

- **Embryonic stem cells (ESCs),** derived from the inner cell mass of the blastocyst; ESCs are pluripotent, representing the descendants of totipotent cells, they give rise to all the cells in the adult organ. According to different physiological conditions, ESCs form specialized cells of all three germ layers: *endoderm* (stomach and gastrointestinal lining, and lungs), *mesoderm* (muscles, bones, blood, and urogenital system), and *ectoderm* (epidermal tissues and neuronal lineages, as neurons, astrocytes and oligodendrocytes). Due to their extensive cell renewal ability, under different environmental stimuli ESCs can generate several lineage-committed progenies, generally maintained during the lifespan of organism. The potentially unlimited

capacity of self-renewal and plasticity have been considered the bases for the development of embryonic stem cell therapies. In particular, ESCs have been proposed for regenerative medicine and tissue replacement after injury or disease.

- **Adult stem cells**, comprising somatic stem cells (SSCs) and germ stem cells (responsible for spermatogenesis and oogenesis).
    - SSCs are responsible for tissue homeostasis, and regeneration after injury of the organ in which they reside, are multipotent (able to generating multiple differentiated cell types of a particular tissue/system) or unipotent [130]. SSCs are present in tissues with both high (skin, bone marrow, etc.) and low cell turnover (such as heart and blood vessels).

Clonogenic assay enable stem cells isolation through the ability of single cells to differentiate and self-renew [131, 132]. Nowadays, researchers try to define a distinctive set of cell surface markers.

Of great interest is also the characterization of the stem cell niches, specialized microenvironments involved in the preserving cell stemness and providing the stimuli for their activation to generate differentiated cells. In adults, stem cell niches are different from each other as far as cellular composition, structure, and localization. The balance between self-renewal and differentiation is allowed by the conservation of biological properties through a specific interaction between stem cells and their niches.

Alterations in the niche cellular microenvironment are critical for tissue homeostasis and have been involved in the trigger of the oncogenic process.

1.3.1 Cancer stem cells

Stem cells are a key element not only for developmental biology and regenerative medicine, but also in cancer biology.

Tumor contain cell subpopulation at variable state of differentiation giving high intra-tumor heterogeneity: a major subpopulation of differentiated tumor cells forming the tumor bulk; and a smaller subpopulation of undifferentiated CSCs self-renewing and able to differentiate. These two cell subsets show different gene expression, tumor propagation potential, and response to therapy, likely due to genetic and epigenetic modulations, metabolic features and microenvironmental factors [133].

The traditional theory of carcinogenesis based on the stochastic hypothesis, in which the same tumorigenic potential is maintained by all cells within the tumor mass, has been replaced by the hierarchical model after the CSC detection, isolation and characterization [134, 135]. CSCs may derive from normal SSCs after genetic alterations and/or from microenvironmental stimuli [136]. CSCs sustain the malignant properties of tumors, including chemo- and radio-resistance, aberrant regulation of cell survival, proliferation, differentiation, migration, and tumorigenic activity. In fact, several different studies demonstrate that only CSCs are able to initiate tumor in immuno-deficient mice.

In malignant tumors, recurrence is due to the presence of CSCs that play an essential role in tumor maintenance, metastasization and drug resistance [137, 138].

The role of CSCs in cancer has been described in hematologic malignancies, head and neck carcinomas, brain tumors, colon cancer, melanoma, liver, prostate, and ovarian [139, 140]. The definition of CSC is challenging since purification and characterization methods have limitations therefore CSCs are functionally defined by their ability to self-renew and differentiate, and form a tumor which recapitulates the hierarchical cell structure and heterogeneity [141].

Tumor microenvironment, containing different cell types including CSCs (niche) is gaining a relevant role for CSC survival and proliferation; each tumor niche although composed by specific cell subset shares some cell phenotype, epigenetic regulation and signaling pathways, modulating CSC-niche reciprocal crosstalk [136].

CSC *model* has led to novel therapeutic goals in to safely eradicate CSCs without affect normal SSCs.

1.3.2 CSC markers

Although in the last years CSCs have been successfully characterized in several human and animal tumors, a specific population of CSCs within a given tumor, showing homogeneous cellular and molecular properties has not been identified yet. The lack of specific CSC markers contributes to the still elusive characterization of CSCs; therefore, currently only a panel of putative markers that enrich for stemness different tumors is available. The expression of stemness markers, such as NANOG, OCT4, and SOX2, have been reported in different tumors such as oral squamous cell carcinoma[142] [142, 143] prostate cancer [143], and breast cancer [144].

An in-depth study of the molecular mechanisms involved in the proliferation of CSCs and the detection of selective markers allowing the identification of this subpopulation, are important goals for the investigation of new possible therapeutic targets.

Recently, CSCs and their functional role have been also studied in benign tumor, suggesting that also benign tumor initiating cells are endowed of niche-dependent plastic functions, regulated by epigenetic events and signaling pathways. A cell subpopulation within both hormone-producing and non-functioning pituitary adenomas, characterized by self-renewal ability, and expressing neural stem cell marker such as nestin, CD133, DCX, TUJ-1, and Musashi-1, was proposed to be responsible for benign tumor initiation and maintenance [145].

In particular, a subpopulation of $CD133^{+}$-cells, selected by growth in stem-cell permissive medium, has shown persistence of self-renewal, high proliferative potential and *in vivo* tumorigenic activity [146].

The expression of CD44 and CD54 in lipoma cell lines explained the ability of local tumor progression and the rich neo-angiogenesis development [147]. Still in lipoma tissue, CD34 positivity is combined with higher replicative ability and maintenance of cell immaturity, acting on stroma formation and synthesizing and remodeling the extracellular matrix [147].

CD34 cells also promote angiogenesis through the secretion of VEGF, PDGF and IL-8 [148, 149]. In hemangiomas, the most common benign vascular tumor of childhood, characterized by rapid growth and slowly spontaneous involution, several studies suggested the presence of tumor proliferating cells expressing stem markers such as CD29, CD44 and CD105, and mesenchymal stem cells [150-152].

1.3.3 CD105 as a putative meningioma stem cell marker

Several putative stem markers have been described in the isolation and characterization of tumor stem-like cells from human meningioma such as CD133 [153-156], stage-specific embryonic antigen-4 (SSEA4) [155], Sox2 [157] and CD44 and nestin [158]. Self-renewal ability, spherogenesis, and *in vivo* tumorigenesis was also reported [159].

These studies open the possibility that also in meningioma a cell subpopulation endowed with stem-like properties could be at the basis of the tumor generation, progression and recurrence in those forms with atypical clinical behavior. Although definite stem markers for meningioma are still lacking, CD105 expression has been proposed as potential target to identify the meningioma subpopulation responsible of the benign tumor recurrence.

CD105, also named endoglin, is an 180kDa homodimeric transmembrane glycol-protein, belonging to the TGF-β receptor complex. CD105 is significantly expressed in condition in which angiogenic activity is highly activated, such as in renewing of inflamed tissues, or in tumors. Many evidences show that CD105 expression is up-regulated by hypoxia [160] and, in solid tumors, anti-CD105 immunoreactivity tightly matches with activated endothelial cells within peri- and intra-tumor vessels involved in tumor neo-angiogenesis, as also observed in meningioma. On the contrary CD105 expression is absent or very weak in the vascular endothelium of normal tissues [161-163].

CD105 was proposed as putative renal cell carcinoma stemness marker [164] which confers self-renewal ability and concurs to chemo-resistance in human kidney cancer [165].

However, CD105 expression was also reported in human meningioma. In particular, in rhabdoid meningioma CD105-positive cells had a significant proliferative activity and self-renewal ability and co-expressed surface mesenchymal progenitor cell markers (vimentin and SOX2) [166, 167].

CD105 could represent a specific marker for detection of neo-vessels in meningioma, in comparison to pan-endothelial markers and its presence is significantly correlated to the growth fraction and histological grade of meningiomas. Importantly, it has been proposed that the levels of CD105 could have a prognostic impact on the overall survival and recurrence risk of this neoplasia.

1.4 CHEMOKINES

Cellular physiological processes, as survival, proliferation and differentiation are mediated by both direct cell-cell (or cell-matrix) interactions and secreted signaling molecules (e.g. growth factors, hormones, cytokines, chemokines).

Signaling by secreted molecules can be classified according to the distance over which the signal acts, as

1. autocrine: the cell produces a factor which stimulates itself;
2. paracrine: the factor secreted by one cell type affects adjacent cells;

3. endocrine: the secreted molecule via the circulatory system acts on distant cells.

In general, these integrated signals lead to the stimulation or repression of gene expression, by binding to intracellular or membrane receptors expressed by target cells. Among signaling molecules, chemokines are a family of small chemotactic cytokines (8-10 kDa) produced by cells and secreted into the extracellular space to guide cell migration, particularly of lymphocytes [168].

The term chemokine derives from *chemotaxis* (directional movement of cells or organisms in response to chemical gradients [169, 170] and the Greek "-*kinos,* movement".

Firstly, chemokines were discovered as chemoattractant of immune cell, such as granulocytes, neutrophils and eosinophils, monocytes and lymphocytes. Approximately 50 proteins with a highly conserved monomeric structure belong to the chemokine families, and more than 20 known receptors interact with them [171].

According to their amino acid sequence, chemokines have been classified into four subclasses based on the position of their two N-terminal cysteine (C) residues: C (with a single cysteine residue); CC (with two adjacent cysteines); CXC (with a variable amino acid between cysteines), and CX_3C (with 3 variable amino acids between cysteines) [172, 173].

Cysteine conserved motif imposes the formation of two disulfide bridges [174] and structural characteristics are related to chemokine functions [175]. CC chemokines generally recruit eosinophils, T cells, monocytes, basophils, natural killer (NK) and dendritic cells (DC); CXC chemokines attract polymorphonuclear cells such as

neutrophils [176]. C chemokines have only two members XCL1 (lymphotactin-α) and XCL2 (lymphotactin-β). CX_3CL1 (fractalkine) is the only member of the CX_3C group and it is secreted and linked to the surface of cells with chemoattractant and adhesion molecule roles.

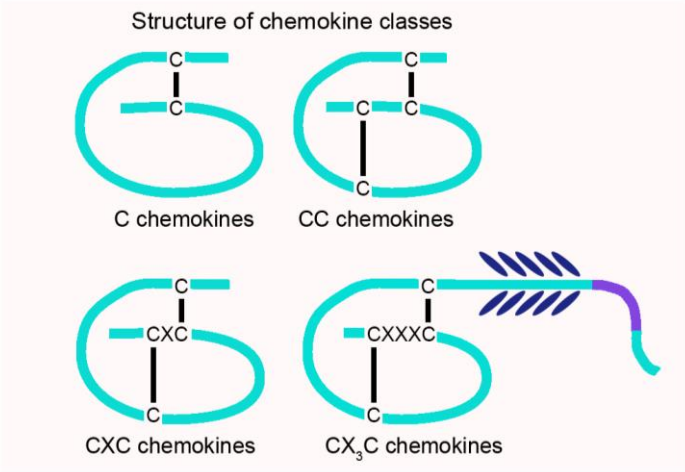

Figure 2 Structure of the 4 chemokine classes: all chemokines have peptide chains (in light blue) and disulphide bridges (in black). CX3C chemokines have also a hydrophobic domain (in purple) with mucine-like domain (in blue). Figure adapted from Wikipedia: https://en.wikipedia.org/wiki/Chemokine

Chemokines and their receptors are promiscuous and redundant; many chemokines usually interact with several receptors, while some receptors can bind different chemokines.

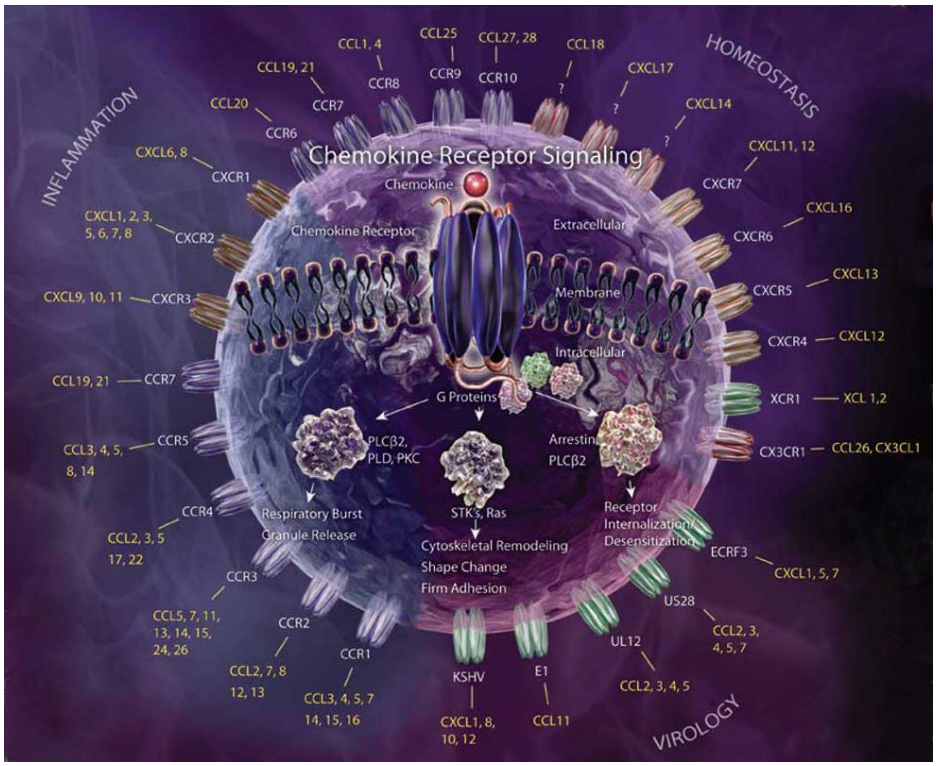

Figure 3 The chemokine universe: chemokines and chemokines receptors families. Figure adapted from BioLegend.com

Chemokines play a key role in a variety of physiological processes, in tissue development and homeostasis, and also are involved in the pathogenesis of tumors, supporting angiogenesis, cell migration, and metastasis. Linked to tumor development or viral infection, an up-regulation with modulation of proliferation, survival and migration has been described. In fact, cancer cells seem to use chemokine-induced chemotaxis to home in on specific tissues.

1.4.1 Chemokine receptors

Chemokines bind transmembrane receptors belonging to two families: conventional chemokine receptors (CCKRs), coupled to G-proteins (GPCR) for signal transduction and atypical chemokine receptors (ACKRs). To date 18 CCKRs have been identified in

humans, whose nomenclature is based on the predominant bound chemokine type (i.e. 10 CCR, 6 CXCR, 1 CX3CR, 1 XCR). To date 6 ACKRs have been identified: ACKR1, ACKR2, ACKR3 (previously named CXCR7), ACKR4, ACKR5 and ACKR6 [177].

Human chemokine receptors usually contain approximately 340-370 amino acid residues, a short N-terminal end, seven helical transmembrane domains, and an intracellular C-terminus with serine-threonine residues for phosphorylation due to the interaction with ligands.

Chemokine receptors present a sequence homology ranging from 25 to 80% at the amino acidic levels. Although they have highly similar primary sequences, they typically link to a limited number of ligands.

Table 5 Chemokines and receptors classification

Systematic name	Common name (acronym)		Receptor(s)
C Chemokines			
XCL1	Lymphotactin-α	*Lymphotactin-α*	XCR1
XCL2	Lymphotactin-β	*Lymphotactin-β*	XCR1
CC Chemokines			
CCL1	I-309	*I-309*	CCR8
CCL2	MCP-1	*Monocyte chemoattractant protein -1*	CCR2
CCL3	MIP-1α	*Macrophage inflammatory protein-1α*	CCR1
CCL4	MIP-1β	*Macrophage inflammatory protein-1β*	CCR1, CCR5
CCL5	RANTES	*Regulated on activation normal T cell expressed and secreted*	CCR5
CCL6	C10		CCR1
CCL7	MCP-3	*Monocyte chemoattractant protein-3*	CCR2
CCL8	MCP-2	*Monocyte chemoattractant protein-2*	CCR1, CCR5
CCL9/CCL10	MRP-2		CCR1
CCL11	Eotaxin	*Eotaxin*	CCR2, CCR3, CCR5
CCL12	MCP-5		
CCL13	MCP-4	*Monocyte chemoattractant protein-4*	CCR1, CCR3, CCR5
CCL14	HCC-1	*Hemofiltrate CC chemokine*	CCR1, CCR5

CCL15	Lkn-1	*Leukotactin-1*	CCR1, CCR3
CCL16	LEC	*Liver-expressed chemokine*	CCR1, CCR2, CCR5
CCL17	TARC	*Thymus- and activation-related chemokine*	CCR4
CCL18	PARC	*Pulmonary-and activation-regulated chemokine*	CCR8
CCL19	ELC	*Epstein-Barr virus-induced receptor ligand chemokine*	CCR7
CCL20	LARC	*Liver-and activation-related chemokine*	CCR6
CCL21	SLC	*Secondary lymphoid tissue chemokine*	CCR7
CCL22	MDC	*Macrophage-derived chemokine*	CCR4
CCL23	MPIF-1	*Myeloid progenitor inhibitory factor-1*	CCR1
CCL24	MPIF-2	*Myeloid progenitor inhibitory factor-2*	CCR3
CCL25	TECK	*Thymus lymphoma cell-stimulating factor*	CCR9
CCL26	Eotaxin-3	*Eotaxin-3*	CCR3
CCL27	ESkine	*ESkine*	CCR10
CCL28	MEC	*Mucosae-associated epithelial chemokine*	CCR3, CCR10
CXC Chemokines			
CXCL1	Groα	Growth-related oncogene α	CXCR2
CXCL2	Groβ	*Growth-related oncogene β*	CXCR2
CXCL3	Groγ	*Growth-related oncogene γ*	CXCR2
CXCL4	PF-4	*Platelet-factor-4*	CXCR3
CXCL5	ENA-78	*Epitelial cell-derived neutrophil-activating factor 78*	CXCR1, CXCR2
CXCL6	GCP-2	*Granulocyte chemoattractant protein*	CXCR1, CXCR2
CXCL7	NAP-2	*Neutrophil-activating protein*	CXCR2
CXCL8	IL-8	*Interleukin-8*	CXCR1, CXCR2
CXCL9	Mig	*Monokine induced by γ-interferon*	CXCR3
CXCL10	IP-10	*γ-interferon-inducible-protein-10*	CXCR3
CXCL11	I-TAC	*Interferon-inducible T cell α-chemoattractant*	CXCR3, CXCR7 (ACKR3)
CXCL12	SDF-1	*Stromal cell-derived factor-1*	CXCR4, CXCR7 (ACKR3)
CXCL13	BCA-1	*B cell-activating chemokine-1*	CXCR5
CXCL14	BRAK	*Breast and kidney chemokine*	unknown
CXCL15		*Lungkine*	unknown
CXCL16	SR-PSOX	*Sexckine*	CXCR6
CXCL17			CXCR8
CX3C Chemokines			
CX3CL1	Fractalkine	*Fractalkine*	CX3CR1

Intracellular signaling responses depend on cell types, tissues and, physiologic or pathologic conditions [178]. It has been described that CXCR4, CCR2 and CCR5 form homodimers and, CCR2 and CXCR4 dimerization is necessary for signal transduction and occurs as a result of chemokine binding. Although the redundancy of chemokines-chemokine receptors network each receptor binds almost exclusively ligands of a single subclass. Thus, the chemokine receptors nomenclature is based on the chemokines group to which their ligands belong.

Signal transduction induced by ligand-conventional receptor interaction is mediated by heterotrimeric (three distinct subunits) G_i-proteins and β-arrestins modulating cell adhesion, migration and proliferation. ACKRs act also in a G protein independent manner, and are mainly responsible for chemokine homeostasis, scavenging, gradient formation, and localization mediated by β-arrestins.

Downstream complex pathways are activated by chemokine receptors such as PI3K and JAK/STAT pathways, phospholipase C (PLC) and calcium flux. Moreover, the activation of intracellular pathways such as p38 MAPK, ERK1/2, p21 kinase, Rho family of GTPases, Ras, and NF-kB trigger cell proliferation, survival, differentiation and migration [179, 180].

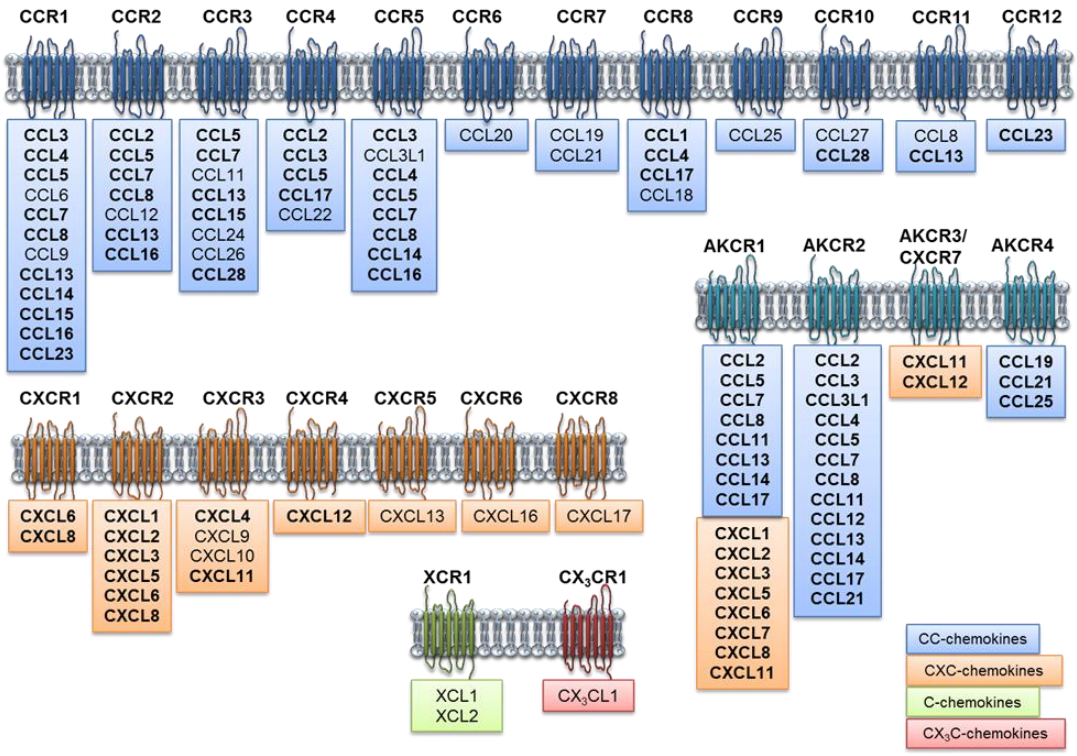

Figure 4 The redundancies and promiscuity of chemokine network. (Figure from Barbieri F, 2016).

1.4.2 Chemokines and tumors

Tumor cells and infiltrating immune cells release pro-inflammatory cytokines, which stimulate the production of chemokines. As result, the over-expression of chemokines and chemokine receptors on the tumor cell surface promotes tumor growth, angiogenesis and metastasis [181, 182]. Moreover, chemokines induce cell-endothelial interactions, promoting transendothelial migration, and enhance tumor cell adhesion and invasion through integrin and matrix metalloproteinases (MMPs) activation [183, 184]. Furthermore, it has been demonstrated that chemokines as CC and CXC regulate immune cell infiltration of the tumor mass [183].

CXCL1 was initially identified as an autocrine growth factor in melanoma cells [185]; its activity is inhibited by specific antibodies or against its CXCR2 receptor, allowing a reduction in cell growth in *vitro*.

CXCL8 also contributes to tumor progression, acting as mitogenic and angiogenic factor, and inducing cell migration. Higher levels of CXCL8 were observed in different human tumors such as malignant mesothelioma, melanoma, and pancreatic, prostate and colon cancers. CXCL8 has also been observed in human pituitary adenoma and neoplastic astrocytes. Anoxia increases CXCL8 expression in gliomas, and the use of CXCL8 asRNA inhibits human glioma cell growth in *vitro* [186, 187]. Studies have demonstrated that CXCL8 to promote endothelial cell proliferation and tumor vasculogenesis, and pro-angiogenic vascular endothelial growth factor (VEGF) and MMP-9 production [178, 188].

Chemokines with ELR motif (N-terminal glutamate, leucine, and arginine-peptide) before cysteine domain such as CXCL1, CXCL5, CXCL7 and CXCL8 present pro-angiogenic activity through the interaction with CXCR2 [189, 190]. Despite the ELR domain absence, CXCL12 is able to induce vascularization. It has been reported that also CCL2 of CC chemokines subgroup acts as direct mediator of angiogenesis [191, 192]. On the contrary, α-chemokines as CXCL9, CXCL10 have angiostatic properties.

In vivo studies showed that interaction between CXCL12 and VEGF induces angiogenesis, and CXCL12 is able to regulate chemotaxis in breast cancer [193].

The expression of CXCL12 plays an essential role in metastasis, and the over-expression of its receptor CXCR4 in the bone marrow and lymph nodes of mice has been observed [194]. A blocking antibody capable of neutralizing the expression of CXCR4 results in a tumor growth arrest [195]. In the last years, the involvement of

CXCL12 in tumor progression has emerged. CXCL12 and CXCR4 have been largely described in several human tumors as glioblastoma and neuroblastoma [196]. In particular, in glioblastoma cell lines CXCR4 is up-regulated and its inhibition reduce cell proliferation [197, 198].

High levels of CXCR4 can be stimulated by cytokines such as TNF-α and IL-1β, present in large concentration in astrocytomas, and in some cases, induced directly from CXCL12. In addition, CXCR4 and CXCL12 have been described in human glioblastoma tissues and their amounts have been correlated with tumor malignancy [197, 199].

Finally, CXCR7 impacts on the tumor formation in knockout mice [200] and, several studies reveal that CXCR7 up-regulation and cellular growth both in tumor cell lines and in human primary tumors.

Although the majority of chemokines are involved in tumor growth, few of them show antitumor activity, inhibiting angiogenesis and activating immune response against the tumor. For example, CXCL9 and CXCL10 act both as angiostatic molecules and T lymphocyte chemoattractant. Interferon-γ induce expression of both CXCL9 and CXCL10 and through an interferon-dependent mechanism, they increase the anti-tumor effects of IL-12 [201].

Moreover, the expression of p53 induces synthesis of the chemokine widely expressed in the CNS, fractalkine, which is able to attract monocytes and cytotoxic T cells demonstrating its involvement in immune prevention of malignant cell transformation [202].

1.4.3 The CXCL11/CXCL12- CXCR4/CXCR7 system in meningioma

CXCR4 and CXCR7 are the two most highly conserved receptors among vertebrates and share the ligand CXCL12. The biological functions of the CXCL11-CXCL12/CXCR4-CXCR7 network have been widely studied in tumors, including proliferation, survival, differentiation, angiogenesis, local invasion and metastasization of cancer cells.

CXCL12 is a CXC chemokine that controls homeostatic processes such as embryogenesis, hematopoiesis, and angiogenesis. At the same time, a higher expression of CXCL12, or of a specific CXCL12 splicing variant, contribute to the onset or progression of diseases [203]. Firstly, CXCL12 was characterized as a pre-B cell growth factor and named stromal cell-derived factor-1 (SDF-1) due to its major natural source in bone marrow stromal cells [204]. CXCL12 acts as mitogenic modulator on its specific receptor CXCR4 for pre-B cells, T cells, dendritic cells, monocytes and hematopoietic precursors; it also supports hematopoietic precursors and CD34+ cell proliferation. However, CXCL12 action is not limited to these cell types, in fact its expression has been extensively described in different organs and tissues including the CNS.

CXCR4 is a GPCR with an extracellular domain for CXCL12 binding. The binding CXCL12-CXCR4 triggers intracellular effects due to the interactions between G proteins and β-arrestin; consequently, adenylyl cyclase is blocked and PLC is activated *via* Src kinase, and PI3K and MAPK pathways activation entails gene

transcription that culminates in migration [184]. At last, CXCR4 is internalized via endocytosis in clathrin-coated vesicles and it can be recycled back to cell surface or, ubiquitinated and degraded [184, 205]. Several organs, including brain, in particular vascular endothelium, microglia, astrocytes and neurons express CXCR4 [175]. CXCL12 and its receptor CXCR4, involved in different human tumor cell proliferation, are expressed both in established meningioma cell lines [206-208] and in primary cultures [167, 209]. Knockout animals for CXCR4 and/or CXCL12 clearly indicated the specificity interaction between these proteins, since the phenotypes of these animals are virtually identical [210-212]. $CXCR4^{-/-}$ or CXCL12 homozygous animals died before birth, demonstrating that these molecules are essential for life in mice [213]. Fibroblasts of meninges release CXCL12, while CXCR4 is expressed by stem cells of the external granular layer. Parallel to the role played in hematopoiesis, SDF-1 is involved in cerebellum development, attracting progenitor cells to their proliferative compartments.

CXCR4/CXCL12 axis is regarded as promoter of tumor growth and metastases, through the inhibition of apoptosis, and promotion of angiogenesis, cellular proliferation and invasion [214]. A pharmacological interference with this network constitutes a promising opportunity to specifically target cancer cells and blood vessels [215, 216].

CXCR7 (or ACKR3) has been identified as receptor for both CXCL12 and CXCL11 (a.k.a. ITAC, shared with CXCR3) [194, 199], acting as a key regulator of the CXCL12/CXCR4 system, by heterodimerizing with CXCR4, and scavenging both CXCL11 and CXCL12. CXCR7 shows a 10-fold higher affinity towards CXCL12 than CXCR4 [211].

CXCR7, like CXCR4 and CXCL12, is widely expressed in fetal and adult SNC tissues and in tumor-associated vasculature and it may have an important role in tumor neo-vascularization [217-220]. Intriguingly, recent observations suggest a critical function of CXCR7 in modulating the activity of the ubiquitously expressed CXCR4 in tumor formation [221, 222]. A diffuse CXCR4, CXCL11 and CXCL12 protein expression has been detected in meningioma tissues, while CXCR7 was mainly present in a subset of tumor vessels [216]. Moreover, the levels of CXCR7 mRNA in meningioma tissues are significantly increased along with higher tumor aggressiveness and a positive correlation between the levels of CXCL11 and CXCL12 was observed [220, 223].

CXCL11 (or ITAC: interferon-inducible T-cell α-chemoattractant) initially described as CXCR3 ligand, shows also high affinity for CXCR7; it is expressed in different cell types such as leukocytes, fibroblasts and endothelial cells, following stimulation with interferon γ (IFN-γ) and interferon β (IFN-β), and to a lesser extent from interferon α (IFN-α) [211, 224]. In particular, it is highly expressed in leukocytes of peripheral blood, pancreas, liver and astrocytes, moderately in thymus, spleen and lungs and in low level in intestine, placenta and prostate [225]. CXCL11 attracts activated T-helper lymphocytes and natural killer (NK) cells, acting on CXCR3 and CXCR7 receptors [211, 226]. It also induces calcium release in T cells activated by the binding to CXCR3. On the contrary, CXCL11 has no activity on unstimulated T cells. CXCL11 plays an important role in CNS diseases involving the recruitment of T cells, and in epidermal immune responses. The interaction between CXCL11 and CXCL12 and their receptors plays also a significant role in the progression and metastasis of various human tumors [227]. As example, a recent study revealed a

significantly higher expression of CXCL11 in colorectal cancer tissue than in the normal colon tissue [228]. Furthermore, Gao Y. et al demonstrated that silencing of CXCL11 inhibits the *in vitro* migration and invasion and in vivo metastatic ability of colorectal cancer cells [228].

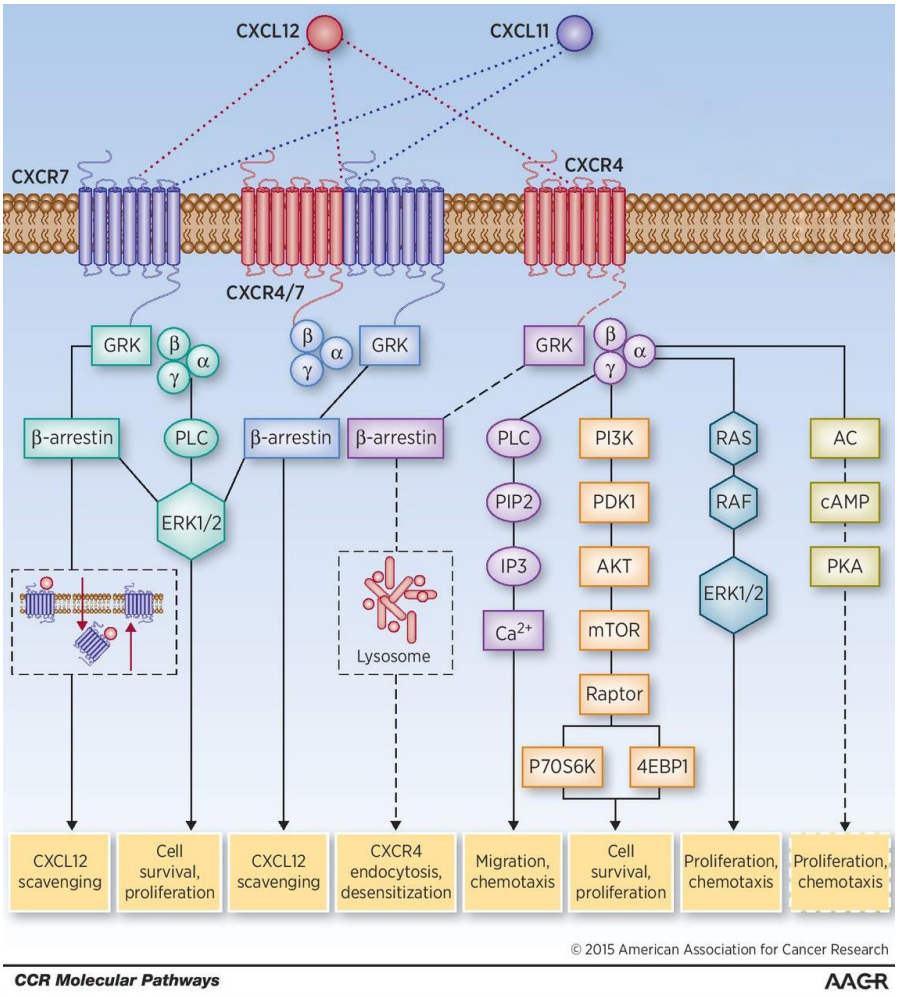

Figure 5 Transduction pathway regulated by the CXCL11/CXCL12- CXCR4/CXCR7 axis. CXCL12 binds CXCR or CXCR7. These two distinct receptors can form homodimers or heterodimers. The binding of CXCL12 to CXCR4 leads preferentially to the association of G_i protein. Cell survival, proliferation and chemotaxis are promoted though MAP and PI3K/Akt intracellular signaling pathway. Activation of CXCR7 or CXCR4/CXCR7 heterodimer or internalized CXCR4 promotes β-arrestin-mediated signaling. Dashed lines show inhibitory pathway as CXCL12 internalization and subject to lysosomal degradation due to binding CXCR4 and CXCR7.

1.4.4 Pharmacological targeting of CXCR4 and CXCR7

As mentioned above, chemokines and their receptors, in particular CXCL11-CXCL12/CXCR4-CXCR7, play a key role in tumor growth, progression and metastasis. On the other hand, they are essential for *in vivo* development and hematopoiesis and, blocking this axis can lead to side effects [181, 229]. Several classes of CXCR4 inhibitors have been developed, among low molecular weight [230]. In the early 1990s AMD3100 (also known as plerixafor or Mozobil®, Sanofi) was originally synthetized to block HIV infection. AMD3100 is a bicyclam, in which two cyclam rings (1,4,8,11-tetraazacyclotetradecane) are linked through an aromatic bridge. AMD3100 acts as a CXCR4 antagonist, and at very high concentrations is an allosteric agonist of CXCR7 [231].

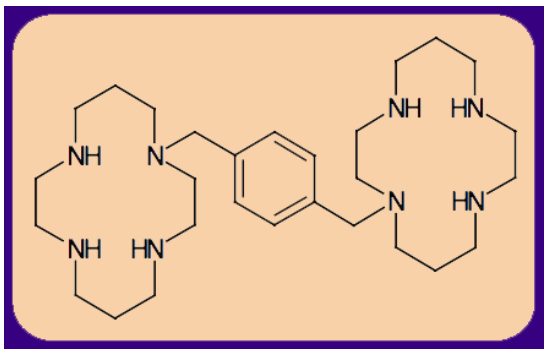

Figure 6 Structure of AM3100

The anti-HIV activity of AMD3100 is due to the block of the interaction of gp120 with CXCR4 during viral entry [232, 233], subsequently the observation from phase I/II studies that this molecule promotes leukocytosis, sustained by the mobilization of hematopoietic cells to the blood, AMD3100 (plerixafor) was approved by FDA and is used to mobilize hematopoietic stem cells from the bone marrow for

transplantation, in patients with hematological cancers such as non-Hodgkins lymphoma, leukemia and myeloma [230, 234]. Moreover, recent studies reveal AMD3100 ability to inhibit CXCL12-induced tumor cell migration *in vitro* and in animal models of breast, ovarian, pancreatic and colorectal cancer, pituitary adenoma, malignant melanoma and glioblastoma [235-237].

To define specific functions of CXCR7, CXCR7-specific small molecule agonists inhibitors of this receptor have been developed for this purpose in recent years, including CCX771, CCX754 and CCX733 (Chemocentyx, Inc.), which compete for CXCL12 binding to CXCR7 at nanomolar concentration, also preventing agonist-induced β-arrestin recruitment [238, 239].

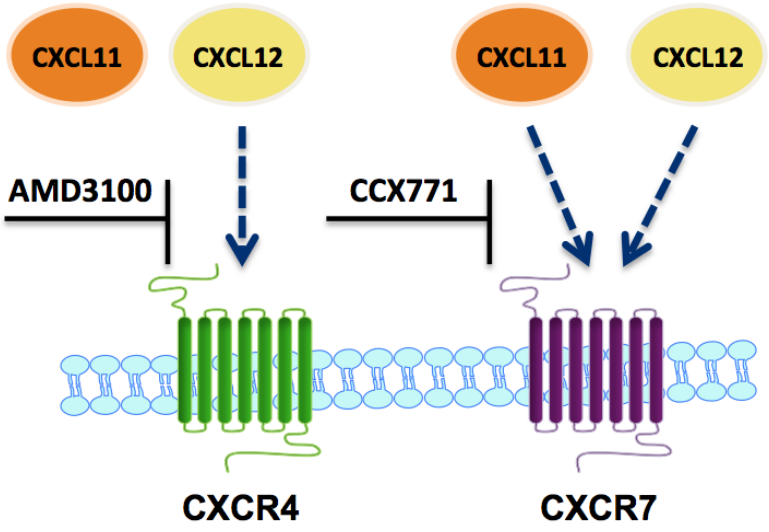

Figure 7 Pharmacological inhibition of CXCR4 with AMD3100 and CXCR7 with CXC771

2. Rationale and Aims of the Thesis

Meningiomas are the most common primary intracranial tumors. They are divided into three grades of malignancy, showing progressive increased risk of recurrence. In most cases, surgical resection is the definitive therapy; however, grade II and grade III have 5-year recurrence rates of about 50% and 90%, respectively, and a significant mortality; in particular, 10-year survival for malignant meningioma is almost 78% for people age 20 to 44 and only about 40% for over 75 years old persons. Importantly, about 20% of grade I tumors also recur in 5 years either for the growth of residual tumor or through the progressing to a higher pathologic grade.

This unpredictable clinical evolution represents a serious therapeutic challenge, since few molecular and biological information are currently available to predict the biological history of the tumor (for example histologically benign meningioma may behave as malignant tumors), and limited treatment options, especially for non-operable and relapsed meningioma patients, have been developed.

Therefore, identification of novel cellular and molecular factors related to and/or determining meningioma aggressive biological behavior and recurrence risk is a major research goal of the current research. Similarly to other brain tumors such as gliomas, meningiomas show high molecular and cellular heterogeneity. The classical theory of cancerogenesis was radically challenged after the cancer stem cell (CSC) identification. Despite the stochastic hypothesis, in which the same tumorigenic potential is maintained by all cells within the tumor mass, a hierarchical model, proposing only CSCs responsible for tumor initiation and progression and for

maintaining tumor heterogeneity, was developed and is now considered the most reliable [134, 135]. CSCs, also called tumor-initiating cells (TICs), are characterized by self-renewal and multi-lineage differentiation abilities like normal stem cells. The presence of stem-like cells plays an essential role in the genesis of several malignant tumors, sustaining tumorigenicity, chemo- and radio-resistance, metastasis, and recurrence.

This new model led to the search for innovative therapeutic approaches aimed to kill CSCs to eradicate the tumor growth.

The definition of CSCs is largely based on functional properties, including self-renewal, ability to differentiate into different tumor-specific cell lineages, drug resistance, invasiveness and migration activity, and, more importantly, the retaining of the tumorigenic potential, as shown by *in vivo* animal experiments. Conversely, the expression of specific stem cell-related markers, often used to select tumor-derived subpopulations does not define CSCs also because phenotypically different CSC subpopulations are present in each tumor.

Recently, CSCs and their functional role have also been studied in benign tumors. In fact, some biological features of CSCs, such as spherogenesis (index of self-renewal) and expression of putative stemness markers (SOX2, NANOG and OCT4), have been identified in benign tumor cell populations; however, to date, the presence of CSCs in meningioma and their contribution to the clinical evolution of these tumors has been scarcely characterized.

Meningioma cells, isolated from human surgical tissues able to form spheres *in vitro* in serum-free medium conditions, and expressing heterogeneous candidate stem markers such as CD133, CD105, CD44, Sox2, and nestin have been proposed as

stem-like cells. Among them, CD105 (cluster of differentiation molecule 105)/endoglin, a transmembrane glycoprotein, has been proposed as a potential stem cell marker in many human cancer, including meningioma.

CSCs reside in the tumor niche, composed of both tumor and normal cell types with diverse functions (immune cells, fibroblast, endothelial cells): CSCs tightly interact with these cell subsets via a complex mutual signaling leading to stemness maintenance, tumor growth, vascularization and invasion/metastasization. Chemokine signaling has a central role in sustaining both bulk tumor cell proliferation and CSC maintenance and functions, driving proliferation, migration and angiogenesis. In particular, the CXCR4/CXCR7-CXCL12/CXCL11 system promotes tumor progression in a variety of solid neoplasms, and CXCR4 is often overexpressed in CSCs. In this context, investigating the supportive activity mediated by chemokine-receptor pairing in CSCs will be crucial to investigate the biology of meningioma stem-like cells and identify new signaling targets for effective therapeutic strategies.

Central goal of the present study has been the identification, the expansion *in vitro* and the characterization of stem-like cells derived from post-surgical specimens derived from human meningiomas, to better understand their contribution to meningioma aggressiveness and the malignant behavior of otherwise apparently benign phenotype of a grade I tumor subset. This aim will be pursued selecting primary meningioma cultures for putative CSCs by growing in stem cell-permissive medium (additioned with EGF and bFGF and devoid of FCS), following the initial protocol used to isolate neural stem cells, and already used in our laboratory to enrich primary cultures of human glioma and pituitary adenoma cell cultures. These

cells will be compared with cells grown in standard 10% serum-containing medium, as *in vitro* model of the "non-stem" differentiated tumor cells composing the bulk of the tumor mass.

Then meningioma stem-like cell subpopulation will be compared with "non-stem" cells for biochemical and biological features ascribed to CSC:

- proliferation rate;
- ability to self-renewal (generation of meningospheres);
- differentiation ability;
- expression of stemness markers;
- vascular mimicry.

Furthermore, I analyzed the proliferative, pro-invasive and pro-angiogenic effects of the CXCR4-7/CXCL11-12 chemokinergic axis in meningioma stem cells as compared to their differentiated counterpart to assess whether a different regulation, likely derived from the niche compartment, between the two populations might occur.

The specific aims of this study can be summarized in the following points:

- Samples collection and *in vitro* expansion of a statistically significant number of primary meningioma cultures;
- Isolation and characterization of putative CSCs in human meningioma;
- Analysis of differential CD105 expression between stem-like and "non-stem" cell populations derived from human meningiomas;
- Evaluation of the expression of CXCL11/CXCL12-CXCR4/CXCR7 system in putative CSCs and differentiated meningioma cells;
- Analysis of the response to the CXCL11 and CXCL12 chemokines of the stem-like and "non-stem" meningioma cells through *in vitro* assays for

proliferation, migration and tube formation;

- Establishment of the individual role of the chemokine receptors CXCR4 and CXCR7 in the above described effects by pharmacological targeting using the antagonists AMD3100 and CCX771, in putative meningioma CSCs.

3. Results and discussion

3.1 Patients and meningioma tissues

During the research activity of my Ph.D. course, between November 2015 and November 2018, thirty-seven consecutive meningioma samples were collected under sterile conditions from the Neurosurgery Unit of the Ospedale Policlinico San Martino – IRCCS of Genova (Prof. G. Zona). Informed consent was obtained from all patients. Soon after surgery, meningioma tissues were divided into two parts: one was formalin-fixed and paraffin-embedded for histopathological diagnosis, and the other was maintained in 0.9% NaCl solution at 4°C, transferred to the laboratory within 2h and immediately processed for cell isolation. When available, a tissue fragment was stored at -80°C for RT-PCR analysis.

Each sample was coded with consecutive number-year of collection to preserve privacy of patients. Diagnosis was assessed at the Pathology Unit of the Ospedale Policlinico San Martino – IRCCS (Prof. J.-L. Ravetti), according to the WHO classification of CNS tumors. Individual clinical and pathological features of patients and tumors are reported in Table 6.

Patient cohort included 2 non-meningeal tumors (1 neurinoma and 1 vascular lesion) which were excluded from further analyses. Proliferative index evaluated by Ki-67 positivity was about 5.1 % (range 0.1-60.0%) in grade I tumors, and 8.7% (range 2.1-15.0%) in grade II.

It is worthy of note that 3 patients showed recurrent tumors, one of these, graded WHO II, recurred with increasing histologic grade from the previous grade I lesion.

Table 6 Clinicopathological characteristics of meningioma patients and tissues

N.	CODE	Sex	Age	Location	WHO grade	Histotype	Ki-67 index (%)	PgR	EMA	Long-term culture
1	**M9-15**	M	42	Posterior fossa	1	M	1.6	5	4	N
2	**M1-16**	F	75	Convexity dx	1	M	3.5	5	3	Y
3	**M2-16**	M	78	Convexity dx	1	M	7.2	5	5	N
4	**M3-16**	F	50	Temporobasal sx	1	M	2.4	5	3	Y
5	**M4-16**	F	54	spinal D5	1	M	3.5	3	1	Y
6	**M5-16**	F	62	Convexity sx	1	M	2.3	3	2	Y
7	**M7-16**	M	60	Convexity dx	1	M	1.4	2	3	Y
8	**M8-16**	F	52	Falcine	1	M	2.5	5	3	Y
9	**M9-16**	F	82	Convexity dx	1	M	1.4	3	2	Y
10	**M10-16**	M	79	Falcine	1	M	4.2	4	3	Y
11	**M11-16**	M	67	Convexity sx	1	An	5.1	3	2	Y
12	**M12-16**	M	65	Orbital roof dx	1	N.D	5.0	4	3	N
13	**M1-17**	F	62	Posterior fossa	1	M	4.1	4	2	Y
14	**M2-17**	M	75	Cerebellopontine angle	1	M	5.8	4	2	N
15	**M3-17**	M	83	Convexity sx	1	M	60.0	4	5	Y
16	**M4-17**	F	58	Posterior fossa	1	M	2.0	4	5	Y
17	**M5-17**	M	65	Convexity dx	1	M	2.7	4	4	Y
18	**M6-17**	F	67	Convexity dx	1	T	2.3	2	4	Y
19	**M7-17**	F	49	Tuberculum sellae	1	M	2.5	4	5	N
20	**M8-17**	F	59	Sfenoid ridge alar	1	M	2.7	4	4	Y
21	**M9-17**	M	64	Convexity°	1	F/P	2.1	1	1	Y
22	**M1-18**	F	68	Sphenoid ridge alar° dx	1	M	8.1	5	4	Y
23	**M2-18**	F	48	Posterior fossa sn	1	T	7.2	5	3	N*
24	**M3-18**	**M**	77	***Neurinoma***						-
25	**M4-18**	F	80	Convexity dx°	2	A	15.0	5	4	Y
26	**M5-18**	M	71	Convexity sx	1	M	3.1	4	4	Y
27	**M6-18**	F	66	Convexity sx	2	A	9.1	5	1	Y
28	**M7-18**	F	59	Tuberculum sellae	1	T	3.9	pos	pos	N*
29	**M8-18**	F	47	Parasagittal dx	1	T	2.5	4	1	Y
30	**M9-18**	F	79	Convexity dx	1	P	2.5	2	3	Y
31	**M10-18**	F	75	Convexity sx	1	F	1.4	2	4	Y
32	**M11-18**	**M**	***65***	***Vascular malfomation***						-
33	**M12-18**	F	53	Convexity sx	1	P	0.1	4	5	Y
34	**M13-18**	F	75	Posterior fossa	1	M	2.1	2	4	Y

35	**M14-18**	M	70	Posterior fossa dx	2	A	2.1	5	4	Y
36	**M15-18**	F	30	Convexity sx	1	M	7.0	4	4	Y
37	**M16-18**	M	47	Cerebellopontine angle sx	1	M	1.2	4	4	Y

Sex: M=male; F=female.
Histotype: M=meningothelial; An=angiomatous; T=transitional; F=fibrous; P=psammomatous; A=atypical; N.D.= not determined.
PgR and EMA immunohistochemical score:1=negative, 2= rare positivity, <5% positive cells, 3= focal positivity (5-20% positive cells), 4= positivity (20-60% positive cells); 5=high positivity (>60% positive cells).
Long term culture: N=no; Y=yes; N*= contaminated.

Among the 35 meningioma patients, 22 were females and 13 males, with a female-to-male ratio of 1.7:1 which is in accordance with literature meningioma gender distribution; the mean age was 63.3 years. All the patients did not receive preoperative treatments.

The majority of such tumors were located supratentorially, commonly in the cerebral convexity (17/35), or in the posterior fossa (6/35) and in the region of sphenoid ridge. As expected, the majority of tumors were WHO grade I (32/35), and only 3/35 were graded WHO II; moreover, the meningothelial subtype was predominant (22/35, 63%), while others were distributed among different morphological subtypes as summarized in Table 7.

Table 7 Overview of meningioma patients and tissues

NUMBER OF PATIENTS	35
MEAN AGE	63.3 years (30-83)
SEX	N.
FEMALE	22
MALE	13
WHO GRADING	
I	32
II	3
HISTOLOGICAL TYPE	
ANGIOMATOUS	1
MENINGOTHELIAL	22
PSAMMOMATOUS	2
FIBROUS	1
TRANSITIONAL	4
FIBROUS/PSAMMOMATOUS	1
ATYPICAL	3
Unknown	1

Overall the tumors entered in our study reflect the histopathologic pattern of distribution expected for human meningiomas, with a great majority of WHO grade I “benign” tumors, known to have an indolent clinical course, but comprising a subgroup of tumors showing an aggressive behavior in which WHO grading did not match with prognosis. Moreover, our patient’s series includes 3 cases graded as WHO II atypical tumors, generally characterized by high variability in recurrence pattern. Overall, beside WHO grade III meningiomas which unfortunately display a nearly 100% 5-year recurrence rate, meningiomas entered our study are grade I and II tumors, which potentially represent challenging tumor types for clinical management. Indeed, histotype, grading, location, extent of resection and proliferative activity, although useful for gross tumor classification, did not help

predicting tumor behavior and recurrence [240]. Currently definite prognostic markers and cellular and molecular characterization are lacking, needed to understand the biological basis of meningioma aggressiveness and identify potential therapeutic target

3.2 Isolation and characterization of putative CSCs from human meningiomas

From November 2015, when I started my PhD in Neuroscience, to 2018, 28 primary cell cultures have been obtained from 35 individual postsurgical specimens of human meningioma collected in the laboratory of Pharmacology directed by Professor Tullio Florio of University of Genova.

In fact, 5 primary cultures from meningiomas which did not yield sufficient number of viable cells and/or long-term cultures and 2 cultures that were contaminated.

Firstly, tumor samples have been mechanically dissociated to obtain single cell suspensions using sterile forceps and scalpels; suspensions were then filtered through a 70 μm strainer to remove possible impurities and fibrous tissue aggregates, plated in DMEM/F12 containing 10% FBS, and allowed to attach to culture flasks. Within 1 week, primary meningioma cultures were obtained and grew *in vitro* as a monolayer (Figure 8).

Medium change was performed at least twice before the experiments to remove red blood cells and cell debris. Cell morphology was observed by an inverted microscope and difference among cultures from different tumor histotypes was detectable (Figure 8): initially, primary cultures appeared as spindle- or round-

shaped cells which progressively attach to the substrate within 2 days, acquiring typical morphologies, reflecting the heterogeneous appearance of meningiomas, after about 1 week: meningothelial meningioma cells (M4-17) comprised a majority of spindle morphologies and some rounded cells; fibroblast-like spindle cells were observed in transitional meningioma cultures (e.g. M7-18, Figure 8) while the psammomatous type (M9-18, Figure 8), was characterized by round groups of cells mixed with branched cells. Cells M4-18, derived from an atypical tumor, possessed some cellular processes and intracellular granularity (Figure 8).

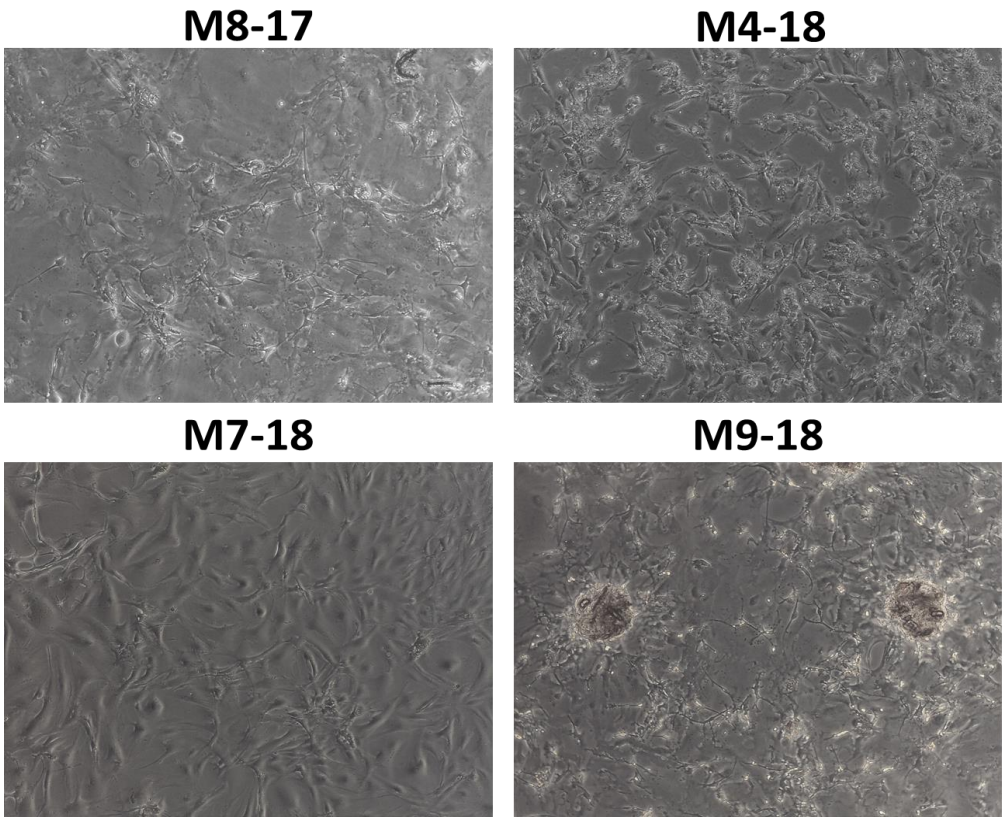

Figure 8 Morphologic characterization of four primary meningioma cultures derived from different histotypes: M8-17 meningothelial, M4-18 atypical, M7-18 transitional, M9-18 psammomatous. Original magnification 20X.

After 1 week in culture, cells were split and maintained in two different media:

1. Stem cell-permissive medium containing growth factors (EGF and bFGF) in the absence of serum, hereafter these cultures were named as "**STEM**";
2. DMEM/F12 FBS 10% medium containing FBS, but devoid of growth factors used for primary cell cultures, allowing them to grow and to differentiate and these cultures are identified as "**DIFF**".

Cells were grown under the two culture conditions for at least 7 days before performing subsequent analyses and experiments. *In vitro*, single-cell suspensions enriched in stem cells and grown in stem permissive medium, usually originated non-adherent spheres, and this culture method is also frequently used to expand both normal and cancer stem cells.

However, these cultures show several limitations, such as sphere size, density and composition heterogeneity, different exposure of surface and central cells to treatments, technical problems to analyze and reproduce functional parameters of floating aggregates. Therefore, to obtain more experimentally manageable STEM cultures, Matrigel-coated dishes/flasks were used throughout our study to grow cells as attached monolayer. Matrigel is an extracellular matrix originally widely used for human embryonic and adult stem cell cultures and successfully used also for CSC adherence, maintenance and long-term proliferation. We found that 0.2% Matrigel, is sufficient for adherence and growth of meningioma STEM cells, and ensures that CSCs retain stemness properties including self-renewal, multilineage differentiation, without entering in the differentiation program as already observed in glioblastoma stem cells [241].

It should be pointed out that even if a large number of primary cultures were obtained, the isolation of meningioma cells presents technical difficulties and these cells are not immortal, often showing limited proliferation over time, and hence the number of cells available could be not sufficient to perform all experiments in the same sample. The same limitations were also observed in the isolation of other stem-like cells from human benign tumors such as pituitary adenomas [146]. Preliminarily, to characterize the phenotype of the cell obtained from tissue disaggregation, a series of markers have been analyzed by Fluorescence Activated Cell Sorting (FACS) analysis in M5-16 derived cells after the first week in culture (passage 0, total), and one week after splitting in the two different culture conditions (passage 1: STEM and DIFF). The panel of analyzed proteins, showed in Figure 9, includes CD105, a marker of mesenchymal cell stemness, previously suggested to be expressed by putative meningioma CSCs, and endothelial (CD31 and CD34) or hematopoietic lineage markers (CD14, CD19, HLA-DR, CD45), and CD106, also known as vascular cell adhesion molecule 1 (V-CAM1).

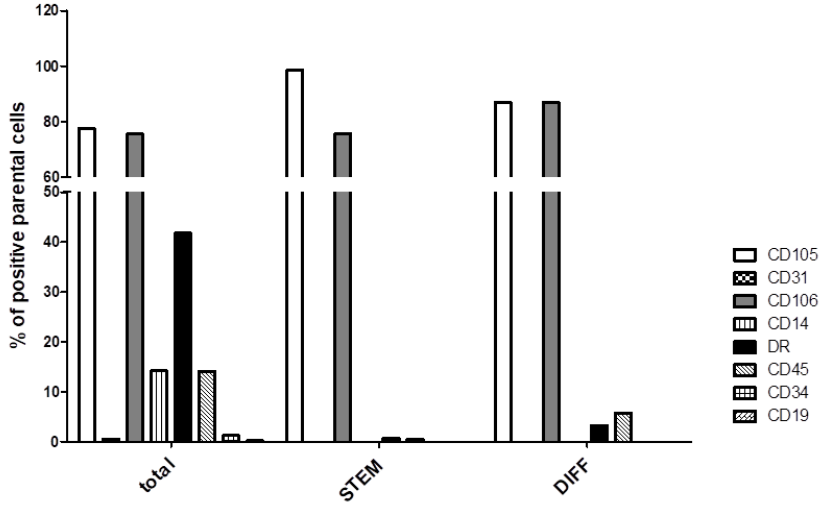

Figure 9. Immunophenotype of meningioma-derived cells at P0 (total) and at P1 in STEM or DIFF medium, by flow cytometriic analysis.

Unselected primary culture contains a great majority of CD105 positive cells (77.5%), often co-expressing CD106 protein (75.5%). Moreover, HLA-DR (41.8%) has also been found, highlighting the presence of antigen presenting cells as macrophages, B-cells and dendritic cells (Figure 9). The expression of other hematopoietic markers, CD14, mainly expressed by macrophages (14.2%), and CD45 presented on all differentiated white blood cells (14.1%), indicate the presence of hematopoietic cells. Endothelial cell markers CD34 (1.3%) and CD31 (0.6%) are expressed in a small number of cells. Overall these data suggest that the primary cultures isolated from the tissue include a large subpopulation of CD105-positrive cells and, as expected still contain some cell types of hematopoietic origin.

After the first subculture (P1) in for a week in STEM and DIFF conditions, hematopoietic markers are no longer present while high levels of CD105 (98.5% in STEM and 86.9% in DIFF) and CD106 (75.5% in STEM and 86.9% in DIFF) positive cells remain in both conditions as reported in Figure 9.

This indicates that further purification of primary cells is achieved, by medium change, sub-culture and selective media, removing unattached cells.

To analyze the morphology of STEM and DIFF cells, cultures have been observed by transmitted light microscope and ZOE™ Fluorescent Cell Imager microscope after staining with Vybrant™ CFDA SE Cell Tracer Kit to detect viable cells (Figure 10).

STEM and DIFF cultures exhibited divergent cell morphologies: in STEM medium cells showed heterogeneous size and shape, with short cellular processes, and lacked remarkable granularity, while in DIFF medium appeared enlarged, flattened cells with granules.

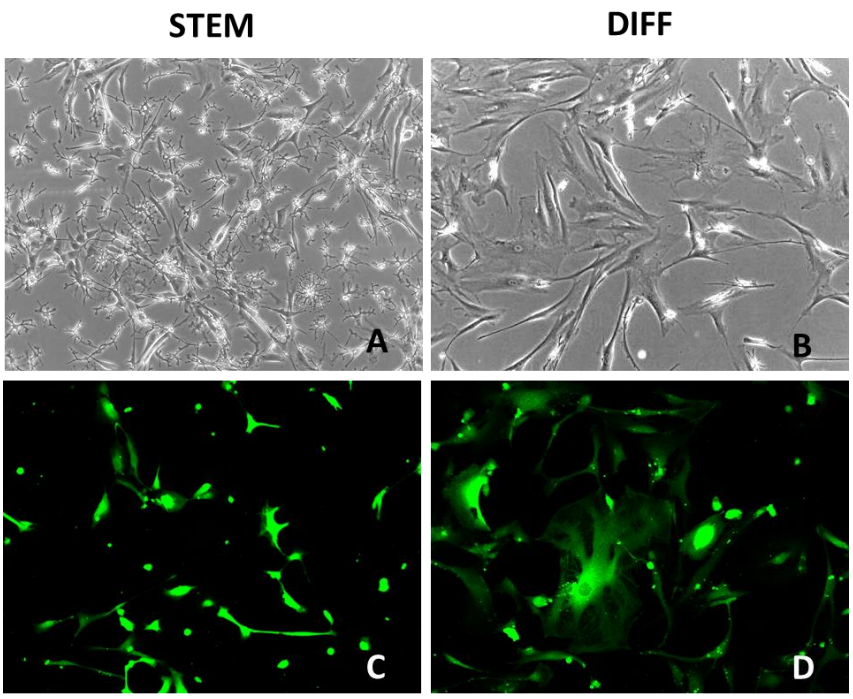

Figure 10. Morphologic characterization of meningioma STEM- and DIFF-cultures. A-B) Representative morphological appearance of STEM- and DIFF cells grown as monolayer (Original magnification 10x). C-D) Representative fluorescent images of viable green fluorescent STEM- and DIFF- cells. Original magnification 10X.

To delve deeper into the features of these meningioma cells, we measured the growth rates of 6 cultures, derived from the same tumors, but differentially selected under STEM and DIFF conditions.

Six cases (M1-18, M4-18, M5-18, M6-18, M9-18, and M13-18) were cultured for 7 days and daily analyzed for cell proliferation, by MTT reduction assay; individual data are plotted in Figure 11A. All tested meningiomas evidenced a significant difference in growth profile when maintained in STEM and DIFF conditions; indeed, starting from the third day, in 5 out of 6 meningiomas, the STEM proliferation was constantly at least 2-fold higher in than the time-matched proliferation of the correspondent meningioma in DIFF medium (Figure 11, panel A). Figure 11 panel B plots results as average values from all the six cultures analyzed.

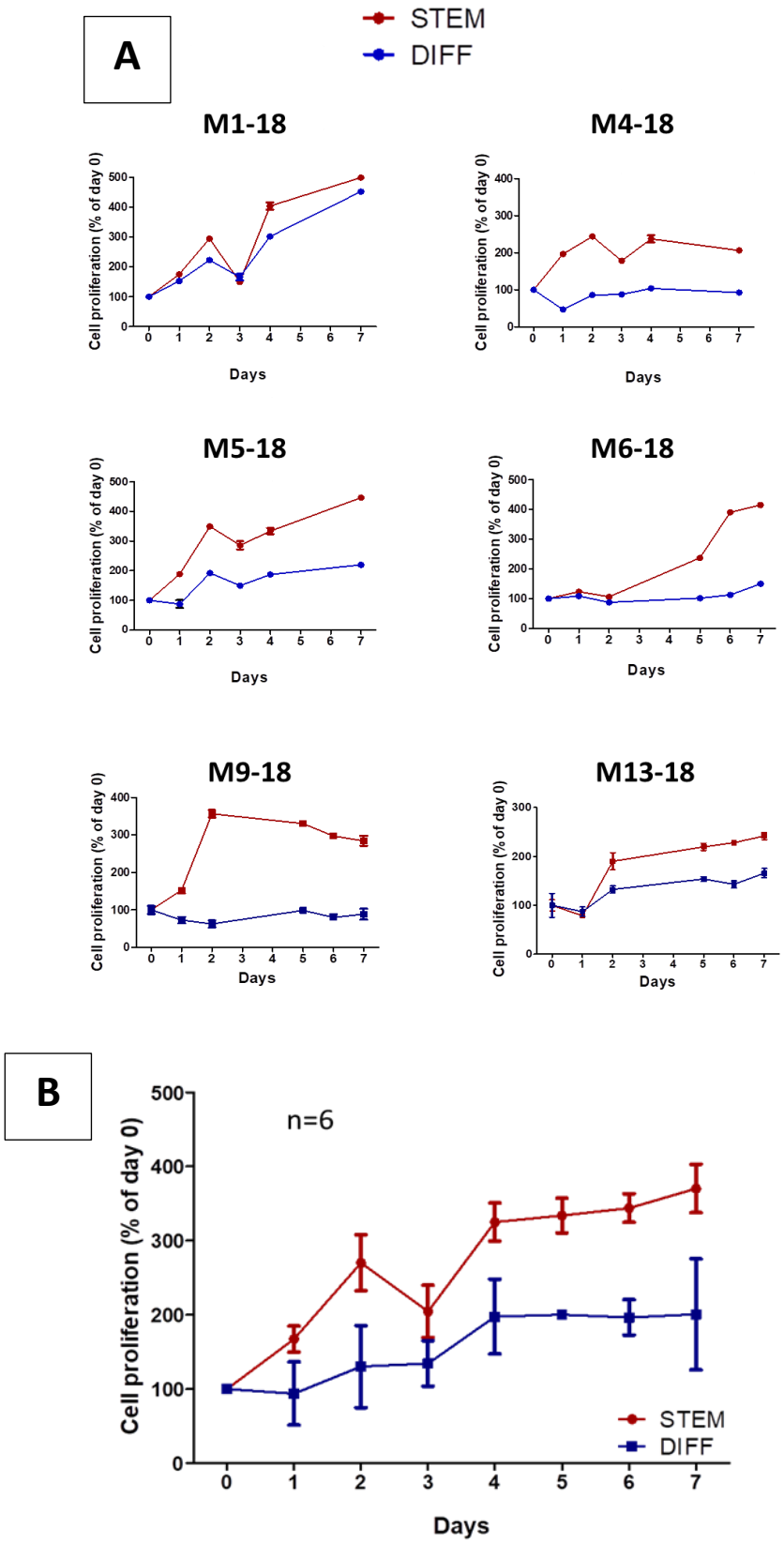

Figure 11. A. Time-course growth curves of STEM (in red) and DIFF (in blue) of meningioma cells assessed daily for seven days by MTT. Each point represents the mean ± S.D. B. Cumulative growth curves representing the mean ± S.D. of 6 meningiomas.

Since medium was changed every three days in culture, we speculate that the reduced differences between the two conditions, that can be observed after three days, is due to growth factors exhaustion in the STEM medium; after medium renewal the difference in proliferation was promptly recovered .It is possible to appreciate that, whereas the stem-permissive conditions allowed a constant growth for up to 7 days at least in 4 out of 6 cultures, under differentiation conditions cell grow slowly was arrested after 2-3 days. These differences are relevant to our study because they support the hypothesis that it is possible to maintain long-term growing stem-like subpopulations from meningioma tissues.

Hence, we investigated more in detail the presence of hallmarks of stemness in both STEM and DIFF cells such as the sphere formation, which represents one of the functional features of CSCs by evaluating self-renewal activity. Spherogenesis was analyzed in 9 primary STEM cultures, plated on Matrigel-free polystyrene dishes and allowed to grow for seven days in STEM medium, and their DIFF counterparts were shifted in STEM medium for the same time. Cultures were monitored daily and at day 7, phase-contrast images were taken to evaluate the presence of floating spheroids (Figure 12).

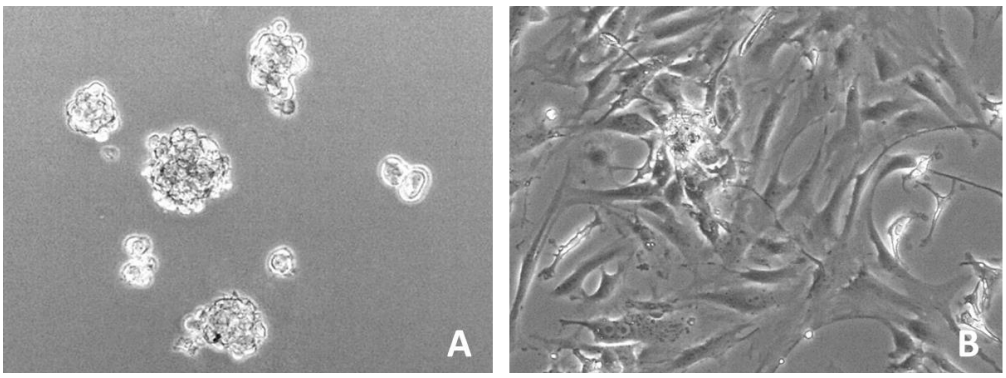

Figure 12 Representative morphological appearance of (A) STEM cells, without Matrigel in STEM medium, forming spheres and (B) cells originally maintained in DIFF medium and shifted in STEM medium, still growing as adherent monolayer. Cultures were observed after 7 days in vitro and photographed by phase contrast microscopy. Original magnification 20X.

Five out of nine STEM cultures grown without Matrigel were able to form meningospheres, spheroids of variable size, shape and compactness, while four of them grew as both sphere-like aggregates and adherent cells. In contrast, almost all DIFF cultures (7/9) even in the presence of growth factors were not able to form spheroids and still adhere to the substrate. Only M1-18 and M9-18 meningiomas formed albeit small, aggregates (Table 8). The latter observation indicates that in these cultures differentiation was not complete and possibly partially reversible, although self-renewal ability was still much lower as compared to STEM cultures, at least as far as spherogenesis assay is concerned.

Table 8 Sphere forming ability of meningioma cultures

	STEM	**DIFF**
	Spheroid	*Spheroid*
M10-16	++	--
M1-17	++	--
M3-17	++	--
M5-17	+-	--
M6-17	+-	--
M8-17	++	--
M1-18	+-	+-
M9-18	+-	+-
M15-18	++	--

++=floating spheroid; **--**=firmly adherent cells; **+-**=mixed cultures with spheroid and adherent cells.

Several markers such as CD44, SOX2, CD44, OCT4, NANOG and CD133 have been proposed to determine and/or mark stemness in solid tumor cell subpopulations, mostly referring to malignant lesion, but also identified in different benign tumors including meningioma [155]. Thus, we investigated the expression of above markers in STEM- and DIFF-cells by immunofluorescence (IF), as shown in the panel reported in Figure 13.

Overall, the current analysis showed that the expression of all putative stemness markers was higher, although not completely homogeneous, in STEM than in DIFF cultures, where only scattered cells were positive for CD44 and OCT4, showing that cell grown in stem cell permissive medium retain, and enrich in cell with CSC characteristics.

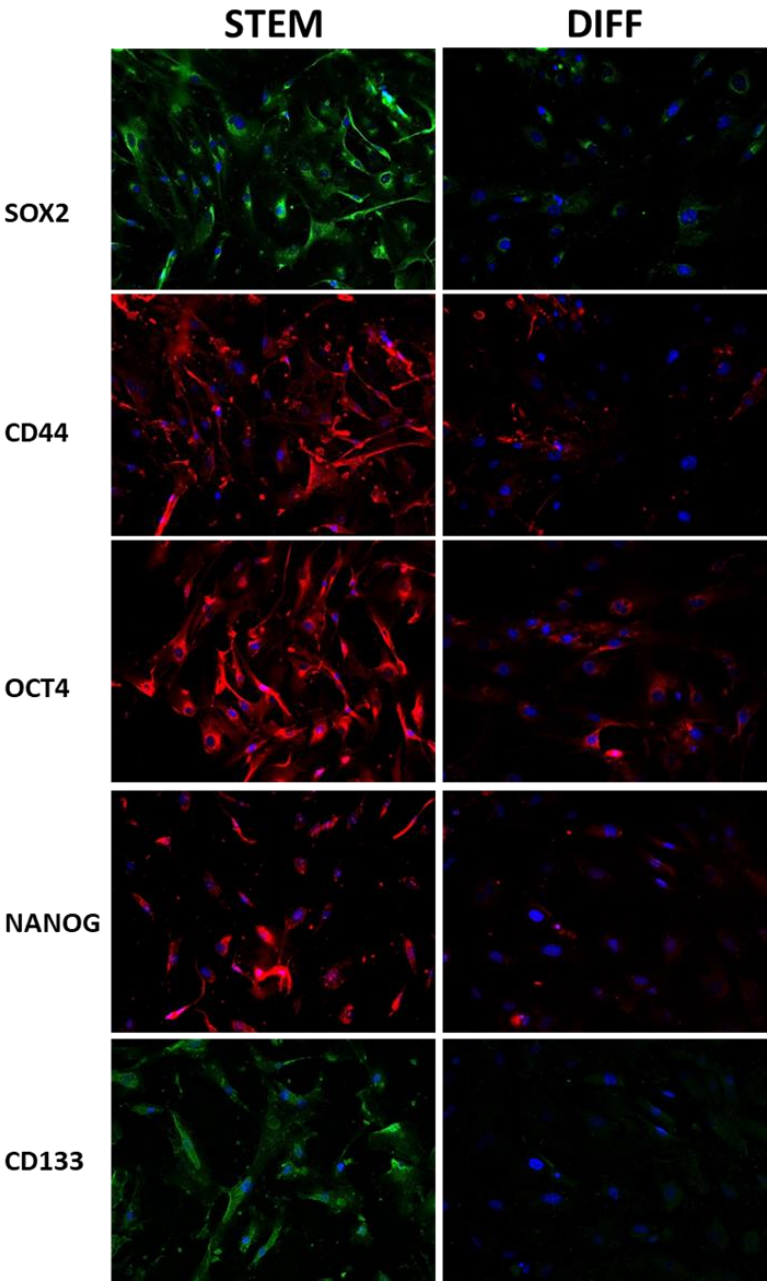

Figure 13 Immunofluorescence analysis of putative stemness markers in STEM and DIFF cells. Representative immunostaining of M8-17 cells for Sox2 (green), CD44 (red), OCT4 (red), NANOG (red) and CD133 (green); nuclei were counterstained with DAPI (blue). Original magnification 20X.

In different tumors, but particularly in meningioma, CD105 expression has been proposed as potential stem cell marker which could allow the identification of the subpopulation of cells responsible for tumor recurrence. In particular, a study performed in rhabdoid meningiomas found a direct correlation between CD105 expression levels and the proliferative activity; moreover, CD105-positive cells revealed self-renewal ability and co-expression of mesenchymal stem cell markers, as vimentin and Sox2 on their surface [166, 167].

On these premises, to delve deeper into the role of CD105 in the two meningioma cell subpopulations we selected, we performed IF analysis in STEM and DIFF cells (Figure 14).

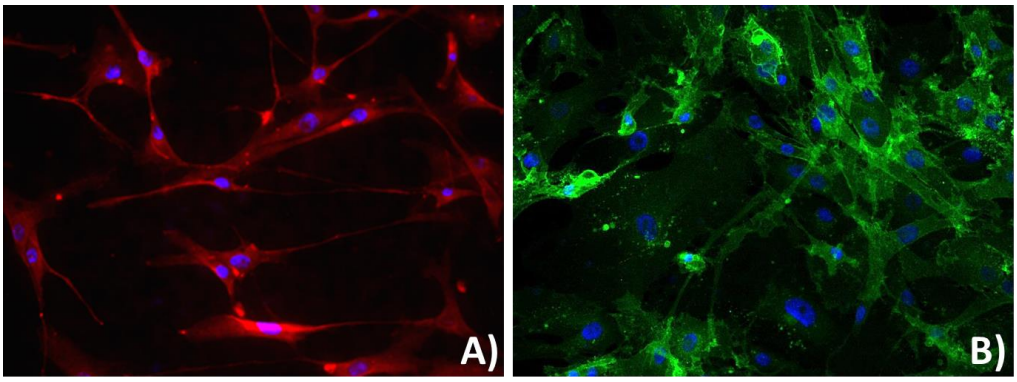

Figure 14 Representative IF staining of primary cultures maintained in STEM (A) or DIFF (B) conditions with anti-CD105 antibody (in red, panel A; in green, panel B) and DAPI for nuclear counterstain (blue). Original magnification 20X.

Unexpectedly, differently from other stem cell markers analyzed, we observed a similar pattern of CD105 immunostaining in cells grown in both conditions, suggesting that, at least in our series of cultures this marker does not define stem-like meningioma subpopulations.

To further verify this result, in parallel with Sox2, CD105 protein level was analyzed by Western Blotting. STEM and DIFF cell lysates from three meningiomas, M8-17,

M1-18, and M5-18, were prepared and WB resulting bands for target proteins and loading controls are reported in Figure15A; densitometric analysis of CD105 and Sox2 protein level reported as ratio of β-actin level, is depicted in figure 15B-D.

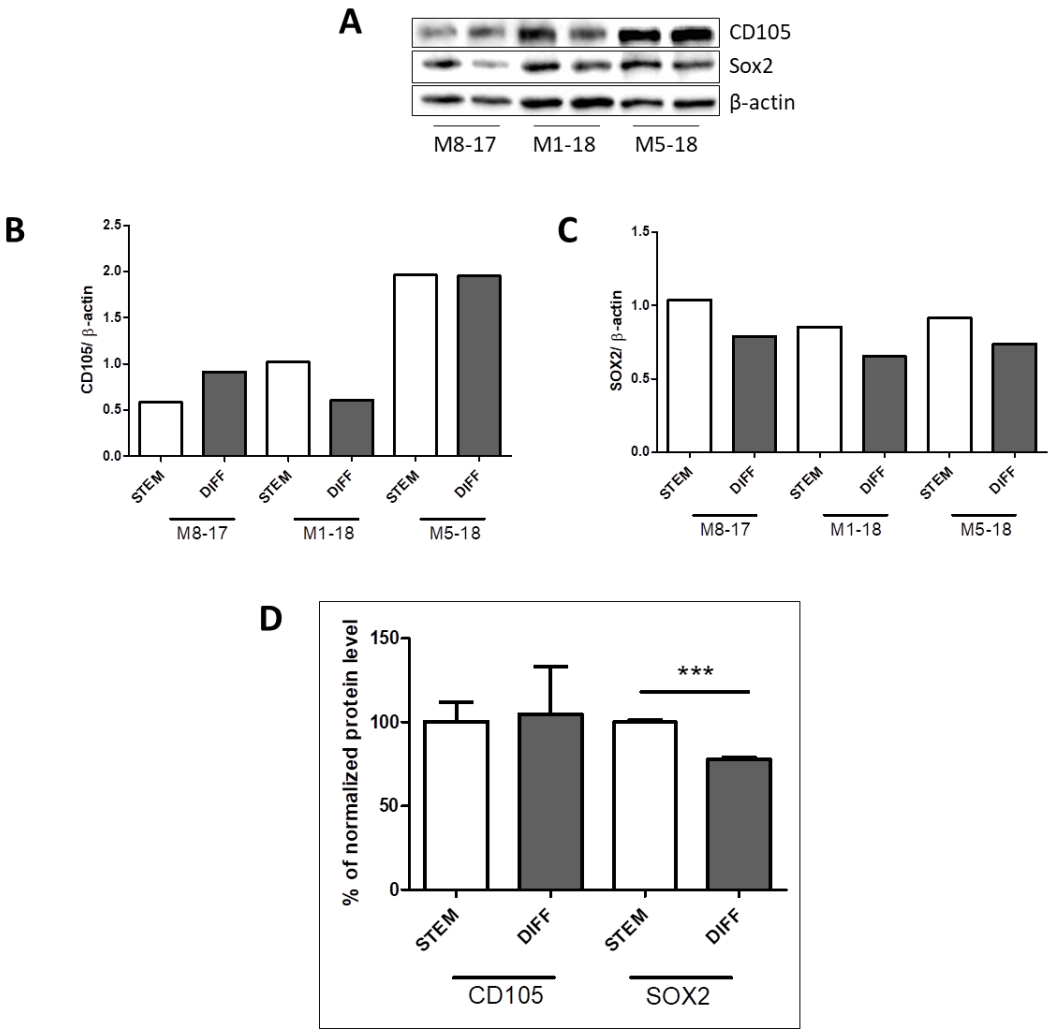

Figure 15. A. Representative immunoblot analysis of CD105 and Sox2 protein levels in 3 STEM and DIFF meningioma cultures. Immunoblot for β-actin was used to normalize the results for the total content of proteins. **B-C.** Quantification of CD105 and Sox2 protein levels, reported as densitometric values of blots in panel A and expressed as CD105/β-actin ratio and Sox2/β-actin ratio. **D.** Mean CD105 and Sox2 levels ± S.D., collecting data from the 3 meningiomas (***p<0.001, t-test).

Heterogeneous CD105 protein levels were observed among different cultures but they did not significantly differ between STEM and DIFF conditions: STEM M8-17 CD105 level resulted lower than in DIFF M8-17 (CD105/β-actin: 0.58 *vs.* 0.91); in M1-18 the amount of CD105 was higher in STEM than in DIFF (CD105/β-actin: 1.02

vs. 0.60), while M15-18 presented approximately equal level of CD105 in both STEM and DIFF cultures (1.95 *vs.* 1.96) (Figure 15B). Interestingly, Sox2 expression was higher in all 3 STEM cultures (Sox2/β-actin: 1.03 *vs*. 0.79 M8-17; 0.85 *vs*. 0.65 M1-18, and 0.91 *vs*. 0.73 M5-18) than in DIFF cells (Figure 15C), reaching a statistically significant difference ($p < 0.01$). The lack of differences in the expression of CD105 in the two meningioma subpopulations we isolated contrasts with the proposed hypothesis of CD105 as putative CSCs marker of human meningiomas.

However, since CD105 is mostly indicated as mesenchymal stem cell (MSC) marker, and the presence of MSCs and their differentiation are critical features in tumor progression, we further investigated the possible mesenchymal potential of the meningioma cultures. To clarify the possible association of MSC features with meningioma progression, the expression of three typical MSC markers, CD105, CD90 and CD73, in both STEM and DIFF cells was examined by FACS analysis in 8 meningiomas. As reported in the histogram of the Figure 16 no significant differences in the percentage of positive parental cells of CD105 antigen between STEM (89.6%) and DIFF (82.5%) have been observed.

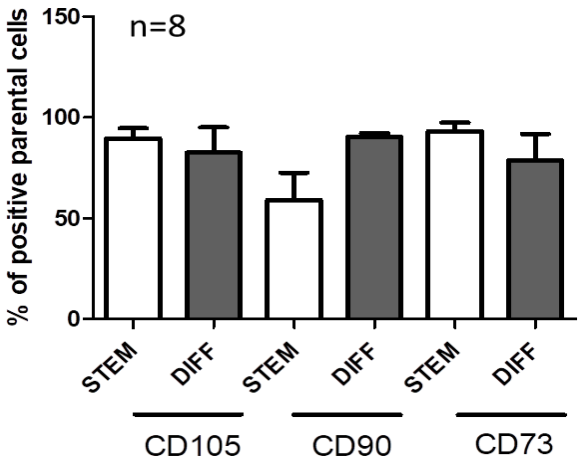

Figure 16 Expression of CD105, CD90 and CD73 mesenchymal markers in STEM and DIFF cells, by flow cytometric analysis.

A higher, although not significant, expression of CD90 antigen in DIFF cells (90.3%) than in STEM cells (59%) was observed and, in contrast, higher level of CD73 was found in STEM *vs.* DIFF cells (93.1% *vs.* 78.6%).

Overall, high co-expression of CD105, CD90 and CD73 surface markers in both STEM and DIFF cells, although at different levels, could indicate the minimal mesenchymal-stem like phenotype of these cells as already reported in grade I meningiomas [242].

Since the ability to differentiate into mesenchymal lineages (adipocytes, osteocytes and chondrocytes), is required to define the MSC type, we investigated the differentiation potential of STEM and DIFF cultures, by culturing cells from four meningiomas in a defined medium for osteogenesis differentiation for 10 days, and calcium deposits were stained with 2% Alizarin Red S.

Slight morphological changes were observed in STEM cells as compared with DIFF cells; in particular, in 2 meningiomas, the increase in calcium deposition, even though relatively low, was evident in STEM cells, as reported in Figure 17A.

To quantify the amount of Alizarin Res S –staining, calcified mineral was dissolved with 10% acetic acid and samples were read by colorimetric detection at 450 nm (Figure 17B).

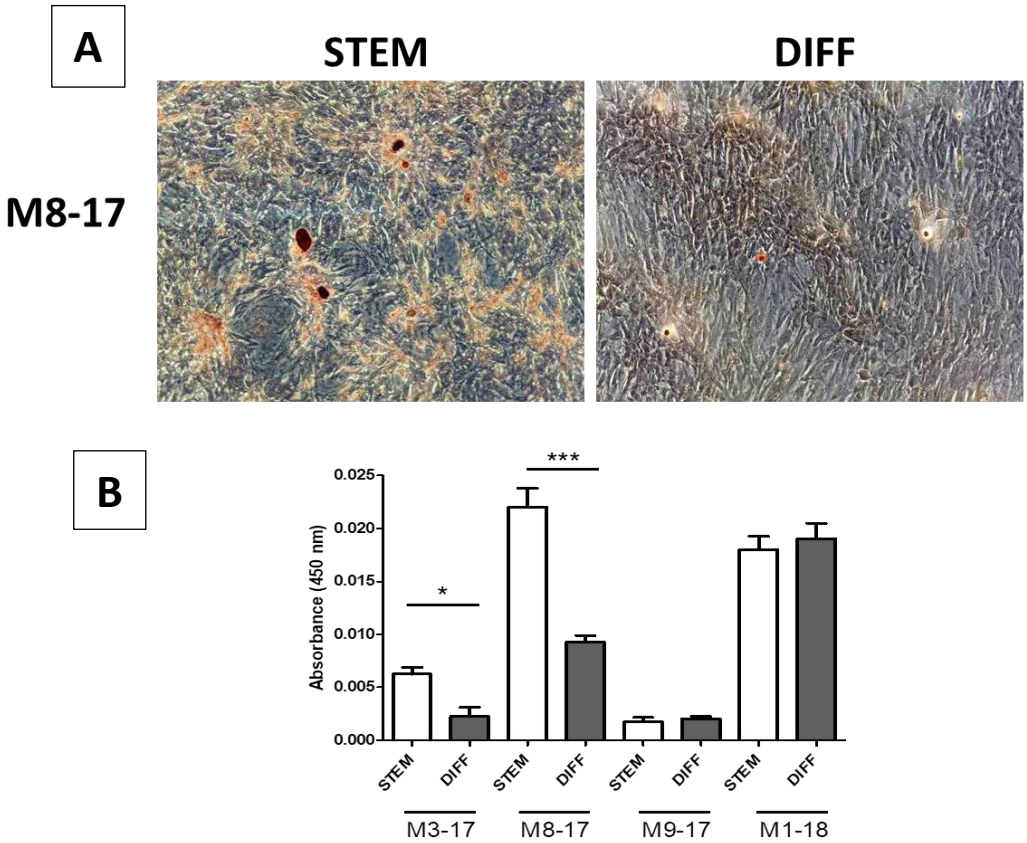

Figure 17. Osteogenic differentiation of meningioma cells. A. Representative pictures of STEM and DIFF cells, stained with Alizarin Red S, from M8-17, maintained in Osteogenesis Differentiation medium for 10 days. **B.** Quantification of osteogenic formation in four meningioma cultures, STEM (white bars) and DIFF (grey bars) (***p< 0.001, *p<0.05, STEM *vs*. respective DIFF cultures, t-test).

STEM cells from both M3-17 and M8-17 meningiomas showed a significant 3-fold higher presence of calcium deposits than respective DIFF cells.

In the same cultures we also evaluated adipogenic differentiation; by maintaining cells in Adipogenesis Differentiation medium for 21 days. The presence of intracellular lipid vacuoles typical of adipocytes, was detected by using Oil Red O staining. Intracellular lipid vacuoles developed only in STEM cells from M9-17 meningioma (Figure 18); while both STEM and DIFF cells derived from the other cultures were not able to differentiate. In only 2 cases in which sufficient cells were available, chondrogenic differentiation was assessed by plating them in the defined

medium; however, neither STEM cells nor DIFF cells were able to adapt and survive the chondrogenic differentiation culture conditions.

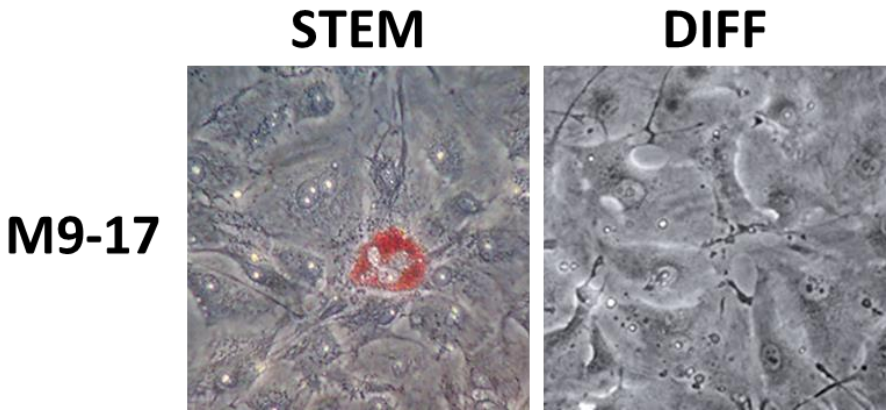

Figure 18 Adipogenic differentiation. Representative pictures of STEM and DIFF cultures from M9-17, maintained for 21 days in Adipogenesis Differentiation medium and stained with Oil Red.

Tri-lineage differentiation seems not consistently occur in our meningioma cultures belonging to grade I tumors, in agreement with Lim et al. (2013) that reported MSC features in cells isolated from meningioma only in few cases (3/10) of WHO II tumors, whereas none of 10 benign meningiomas fulfils MSC phenotyping criteria. Therefore, the role of CD73, CD90 and CD105 proteins in meningioma might be related to other functions, and needs to be further investigated.

To elucidate whether the presence of high levels of mesenchymal markers in meningioma might identify a subset of cells involved in EMT, a process which contributes tumor aggressiveness, we analyzed the expression of epithelial cadherin (E-cadherin) and neural cadherin (N-cadherin) known as key players of EMT. During EMT E-cadherin is downregulated while mesenchymal marker proteins such as N-cadherin and vimentin increase, leading to an invasive and migratory cell phenotype.

Both STEM and DIFF cells expressed N-cadherin, while E-cadherin resulted absent, as detected by IF (Figure 19).

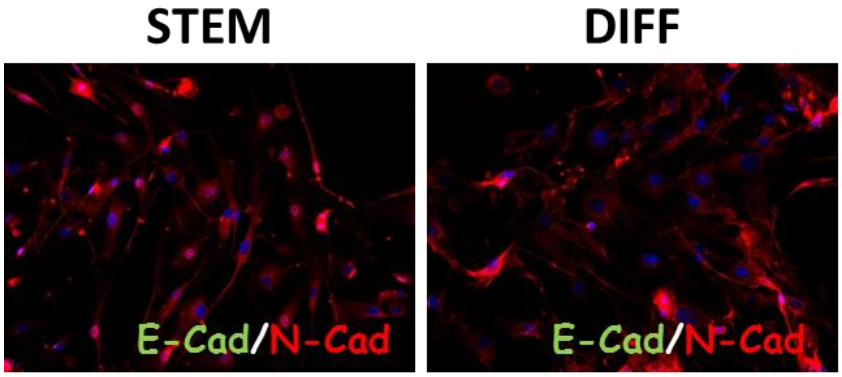

Figure 19 Immunofluorescence analysis of E-cadherin (green) and N-cadherin in STEM and DIFF cells. Representative immunostaining of M8-17 cells; nuclei were counterstained with DAPI (blue). Original magnification 20X.

The expression of E-Cadherin is regulated by transcription factors such as Snail, Twist, thus we performed a quantitative RT-PCR on four meningioma STEM and DIFF cultures to analyze Twist, Snail, Vimentin and E-Cad and N-Cad mRNA levels. Results did not reveal a distinct pattern of expression between STEM and DIFF cells. These results suggest that the different culture conditions did not enrich cells with EMT phenotype, and other factors sustain the distinctive features of STEM and DIFF cultures.

3.3 Meningioma STEM cells display enhanced migration capacity and tube formation

To further characterize the putative stem cell-like biological features of the different meningioma cultures we selected, the migratory and angiogenic (vascular mimicry)

activities were tested in the two populations. The acquisition of migration capacity that allows tumor cells to change their morphology and position, and to pass from a circumscribed and confined tumor to invasive disease, represents a typical feature of cancer, mainly sustained by CSCs, responsible for tumor diffusion and recurrence mediated by cell invasion of extratumoral structures thus preventing the complete surgical removal of all the tumor cells.

We performed trans-well migration assay in 24h-starved STEM and DIFF cells derived from 12 meningioma cultures; in these experiments, cells were labeled with a fluorescent dye prior to seeding in basal medium (serum-and growth factor-free) onto the upper chamber of FluoroBlok™ multiwall insert.

Basal medium, used as negative control, or complete medium containing 10% FBS, used as chemoattractant for cell migration, were added in the lower chamber. Migration through the pores of the membrane at the bottom of the insert, was evaluated after overnight incubation by counting migrated cells (3 microscopic fields for each condition, performed in duplicate) with a confocal microscope.

Overall, although there was some migration in wells containing basal medium in both STEM and DIFF cells, slightly more evident in the STEM counterpart, there was a significant (nearly 5-fold) increase in cellular migration in the presence of FBS, used as chemoattractant, of STEM cells as compared to the correspondent DIFF cells (Figure 20). Importantly, a highly significant migratory activity occurred at shorter timing than the proliferative activity of both cultures, thus allowing a discrimination between the two biological features.

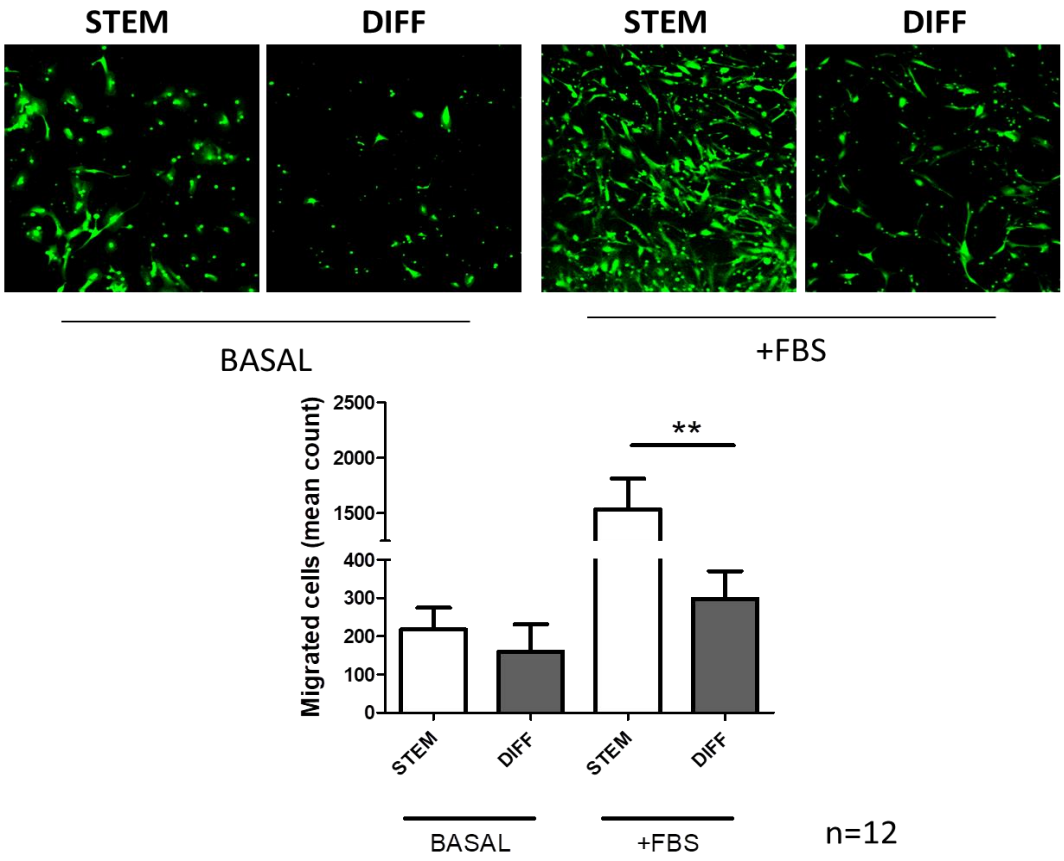

Figure 20. Trans-well migration assay. Representative migration pictures of STEM and DIFF cultures from M1-17 (upper panels). Following o/n incubation, fluorescent cells that had migrated to the underside of the filters were counted on images acquired by confocal microscopy. In the lower panel, histogram represents the mean ± s.e.m. of data from 12 primary meningioma cultures (**p<0.01, t-test).

These results confirm that STEM cells have higher migratory capacity than DIFF cells.

Meningiomas are highly vascularized tumors, and angiogenesis is essential for their growth, likely due to *de novo* vessel formation sustained by CSCs, as previously observed in other solid tumors.

Therefore, we tested the angiogenic activity in 8 meningioma cultures using the *in vitro* tube formation assay, a well-established *test* which evaluates the ability of cells plated in a defined medium, on a gel to form three-dimensional capillary-like tubular structures (vascular mimicry).

The ability of CSCs to differentiate into functional endothelial cells has been largely studied in malignant tumor; in particular, glioblastoma CSCs closely interact with the endothelial niche, determining a directional movement of endothelial cells through the release of chemotactic molecules as vascular endothelial growth factor and CXCL12 in an autocrine/paracrine manner [243-245]. This generation of functional vascular structures contributes to tumor survival altogether with neoangiogenic processes. STEM and DIFF cells were shifted from their respective culture media to Basal EndoGRO medium (hereafter ENDO-BASAL) and, after 24h, were seeded on Matrigel-coated slides and further incubated in both basal medium (negative control) and EndoGro medium containing VEGF (hereafter ENDO-COMPLETE as positive control), formulated to sustain endothelial cell growth in vitro. Cells developing cell-cell and cell-matrix interactions was monitored by phase-contrast microscopy a within 6h tube formation was evident.

As reported in Figure 21, under basal condition STEM cells displayed a slightly higher ability to form tubules than DIFF cells, whereas STEM cells form vessel-like structures more efficaciously than DIFF cells, after exposure to VEGF stimulus.

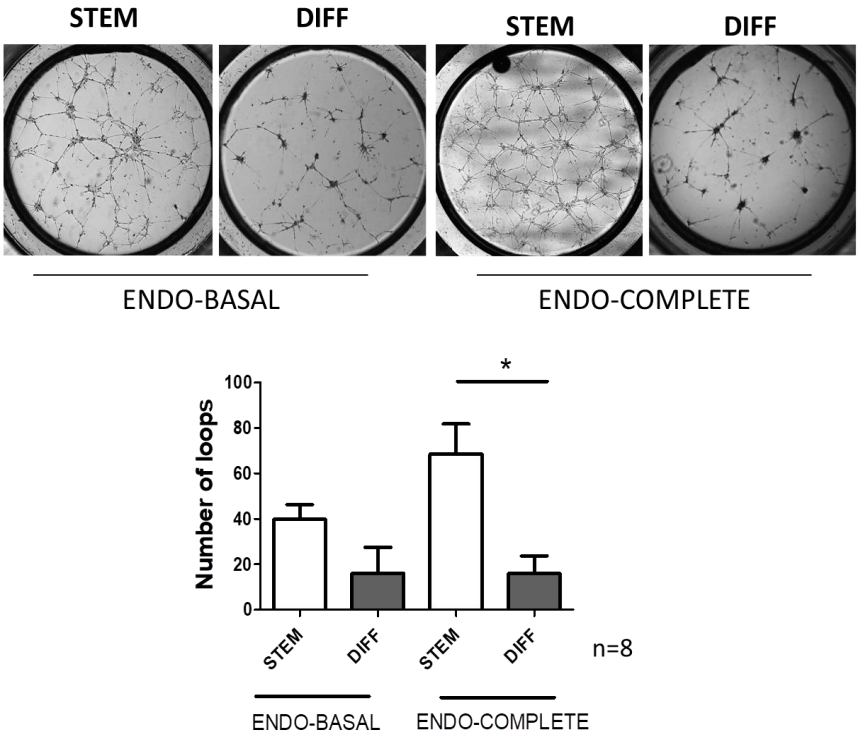

Figure 21 Tube formation assay. Representative pictures of M1-17 meningioma (upper panels). Angiogenesis was quantified by mean count of loop number ± S.D. (*p<0.05, STEM *vs*. DIFF cells, t-test) (lower panel).

In vitro tube formation assay confirm that STEM cells initiate vascularization, undergoing a trans-differentiation process.

As possible molecular correlate of these results, we observed by RT-PCR that STEM cells express 6-fold VEGFR than DIFF cells in 6 meningioma cultures (Figure 22).

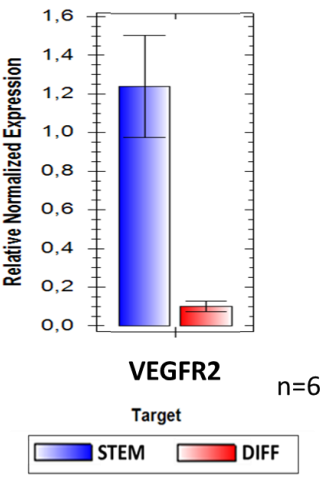

Figure 22 VEGFR2 expression in six meningioma STEM and DIFF cultures. Quantitative RT-PCR analysis showed that STEM cells express at higher level VEGF receptor 2 transcript. Results are given as relative fold increase in mRNA expression. Data were normalized to expression of the β-actin and 28s genes. Bars represent the mean of six distinct meningiomas, ± S.D.

Altogether these results clearly demonstrate that different subpopulations are present within menigiomas that can be differentiated for some of the recognized features of CSCs. In particular, cells selected by growing in stem cell-permissive medium display, as compared to differentiated meningioma cells, 1) higher proliferation rate; 2) ability to self-renew; 3) expression of stem cell markers; 4) mesenchymal differentiation ability (although in this case only few STEM cultures show a differentiation ability); 5) higher migration ability; 6) ability to transdifferentiate in endothelial-like cells.

All these features allow us support the hypothesis that CSC-like cells are present in meningioma and thus efficacious pharmacological therapies should be able to target this population. Conversely, the proposed use of CD105 as a marker for the putative CSC population was not confirmed by our results, since it was expressed similarly in both cultures.

3.4 CXCR4/CXCR7-CXCL11/CXCL12 axis in human meningioma cells

The communication between cells and the tumor microenvironment, the so-called niche, is critical for tumor growth, angiogenesis and metastasis. Several molecules, including growth and chemotactic factors, adhesion receptors, membrane bound and cytokine ligands and receptors, are involved in the regulation of proliferation and self-renewal of CSCs. Moreover, vascular factors induce cell-endothelial interaction, promoting cellular migration and invasion. The main chemotaxis-regulating factors, also protect CSCs from standard therapies as chemo- and radio-

therapy. Pharmacological treatments that act on niche-mediated tumor protective effects could therefore represent a successful strategy against CSCs. In particular, CSCs derived from several human malignant tumors, as well as putative stem cells obtained from benign tumor such as pituitary adenomas [146], overexpress chemokine receptors CXCR4 and CXCR7 [197]. In particular, chemokines and receptors within the CXCR4/CXCR7 – CXCL11/CXCL12 axis are involved in the interactions between CSCs and their niche.

For these reasons, to better understand the biological functions of this chemokine system in human meningioma, we evaluated the expression levels of CXCR4, CXCR7, CXCL12 and CXCL11 in six meningioma cultures, comparing matched STEM *vs*. DIFF cells, by quantitative Real Time PCR (qRT-PCR) (Figure 23).

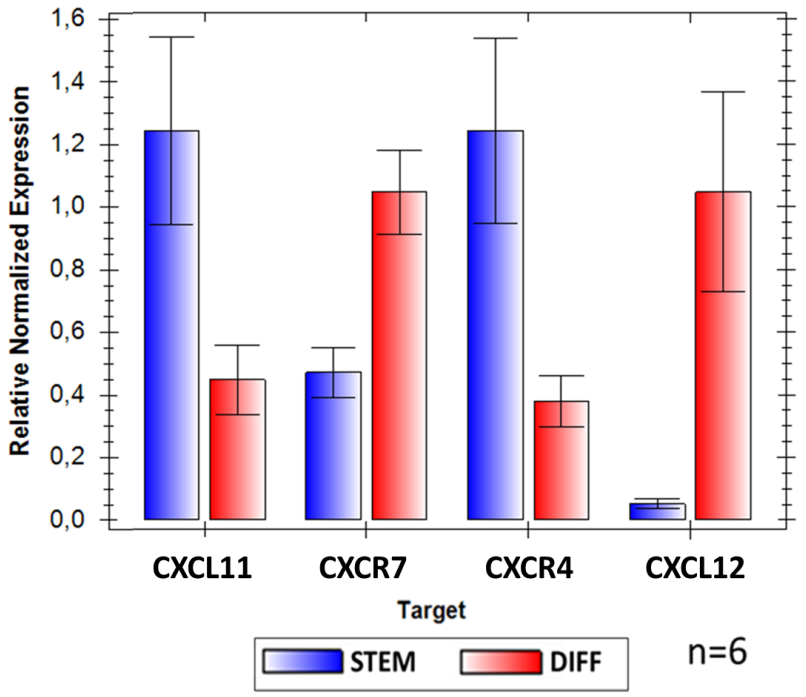

Figure 23 CXCL11, CXCR7, CXCR4 and CXCL12 expression in meningioma STEM and DIFF cultures. Quantitative RT-PCR analysis showed that STEM cells express, at different levels, chemokine and chemokine receptor transcripts. Results are given as relative fold increase in mRNA expression. Data were normalized to expression of the β-actin and 28s genes. Bars represent the mean of six distinct meningiomas, ± S.D.

Interestingly, STEM and DIFF cells express two distinct sets of chemokines and chemokine receptors. In particular, STEM cells preferentially express CXCR4 and CXCL11 mRNA; conversely, in DIFF cells higher mRNA content for CXCR7 and CXCL12 were detected (Figure 23).

Receptors' expression is inversely correlated to that of their ligands, therefore it could be hypothesized a downregulation of the receptors in the subset of cells which highly expressed the respective main ligand. This result suggests that in meningioma STEM cells the CXCL12-CXCR4 system plays a relevant role in stemness and underline an individual role of CXCR4 and CXCR7 in meningioma stem cell population.

On this basis, to expand and validate the results obtained by quantitative RT-PCR, protein levels of CXCR4 and CXCR7 have been investigated using immunofluorescence as displayed in the pictures in Figure 24. DIFF cells seem to be more homogeneously positive for CXCR7, while STEM counterpart showed mainly CXCR4-positive cells but also CXCR7-expressing cells.

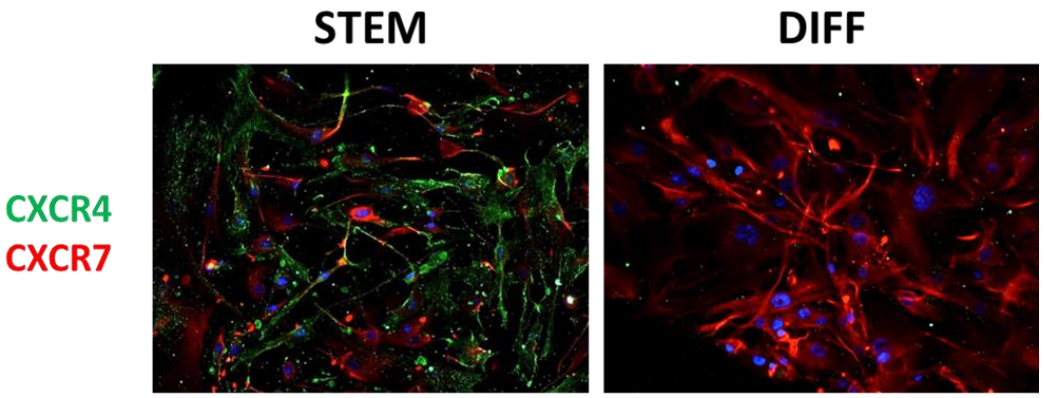

Figure 24 Immunofluorescence for CXCR4 (green) and CXCR7 (red) in STEM and DIFF cultures. Nuclei were stained with DAPI (blue). Original magnification 20X.

3.5 CXCL11 and CXCL12 enhance proliferation of meningioma STEM cultures

To better understand the role of each chemokine in biological functions of meningioma cells, we focused our attention on the modulation of the proliferative potential of STEM and DIFF cultures Cells from meningioma cultures plated in a EGF/bFGF- of FCS-deprived medium for 24h to avoid growth factor interferences, were treated with 25nM CXCL11 or 25nM CXCL12; cells were allowed to grow for further 24h and the effects on cells were analyzed by MTT assay (Figure 25).

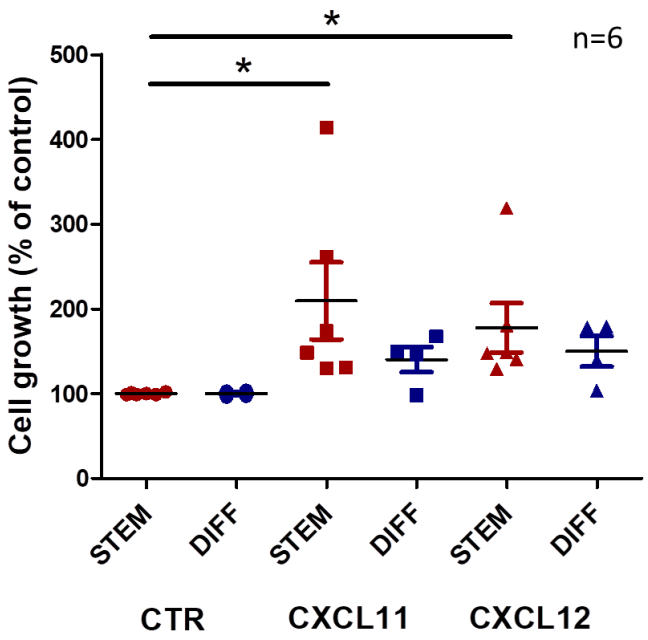

Figure 25 Effect of CXCL11 and CXCL12 stimuli on the proliferation of STEM (red) and DIFF (blue) meningioma cultures by MTT assay. Each point represents the mean value of individual meningioma, expressed as % of respective untreated control cells (CTR), taken as 100%. Horizontal lines depict the mean value of the 6 cases (* $p < 0.05$, *vs*. respective CTR; t-test).

Both CXCL11 and CXCL12 induced significant increase in cell proliferation of STEM cells, while chemokines did not promote proliferation of DIFF cells.

This observation reinforces the hypothesis that not only STEM and DIFF cells express differential pattern of chemokine-receptor mRNAs and proteins, but also the growth of STEM cells, differently from DFF cells, is predominantly regulated through CXCL12 signaling, likely *via* CXCR4. Interestingly, also CXCL11 promotes cell survival and growth, suggesting a cooperation between CXCR4-CXCR7 shared ligands.

3.6 ERK1/2 activation mediates chemokine effects in meningioma STEM cultures

CXCL11 and CXCL12 act via ERK1/2 activation, a crucial intracellular pathway promoting cell proliferation in several cellular subtypes [246]. Thus we investigated CXCL11- and CXCL12-induced signal transduction in six STEM cultures by evaluating ERK1/2 phosphorylation. STEM cells were starved in a growth factor-deprived stem cell permissive medium for 24h and then, stimulated with CXCL11 and CXCL12 at the concentration of 25 nM, for 5 min; then, the amount of phosphorylated ERK1/2 has been analyzed through Western Blot (Figure 26).

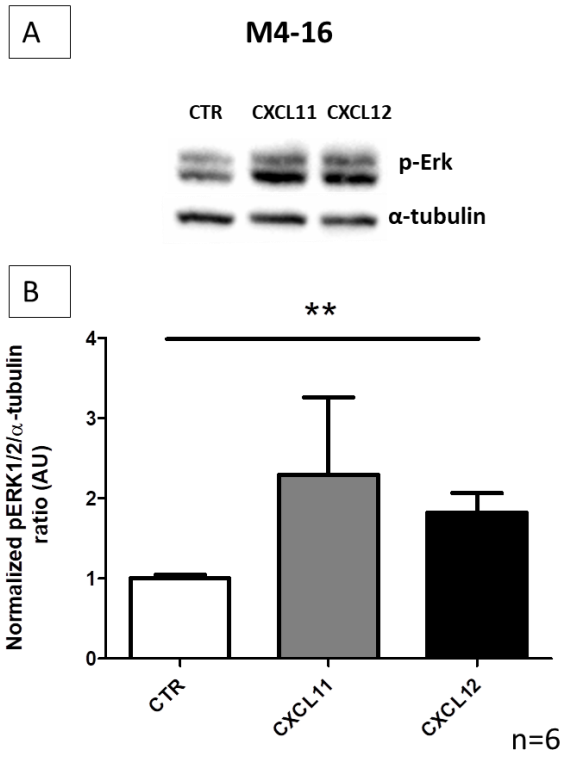

Figure 26 CXCL11 and CXCL12 induce ERK1/2 phosphorylation in meningioma STEM cultures.
A. Representative immunoblot analysis of p-ERK protein level induced by CXCL11 and CXCL12 in M4-16 STEM culture. Immunoblot was re-probed for α-tubulin was used to verify the equal loading of total proteins in samples and normalize the results**. B.** Quantification of p-Erk level by densitometric analysis as mean ± S.D., collecting data from the 6 meningiomas (**p<0.001 *vs*. controls, t-test).

Collectively, we observed that CXCL12 induced a significant increase in phosphorylation of ERK1/2 (about 2-fold) compared to control levels. CXCL11 treatment also determined an increase in ERK1/2 phosphorylation that was slightly higher as mean level (2.3-fold *vs*. controls) than that induced by CXL12, although it was not statistically significant, due to the high variability of the response among the 6 cultures. In fact, the high variability depends on the presence of two meningiomas that did not respond to the CXCL11 stimulus, while CXCL11 induces an increase in phosphorylation of ERK1/2 in the others four meningiomas.

These data confirm that chemokines, in particular CXCL12, play a role in meningioma proliferation, via ERK1/2 activation, likely through an autocrine/paracrine mechanism mediated by CXCR4 and CXCR7.

3.7 Migration of meningioma STEM cultures is potentiated by CXCL11 and CXCL12

Chemokines were initially identified as mediators of leukocyte cross-talk and migration, both in physiological and pathological conditions, and the CXCL12/CXCR4 signaling has been described to play a major role in tumor metastasis and migration in a variety of tumor types. Heterogeneity in the tumor cell population is able to promote invasion and metastasization, as caused by the presence of cancer stem cells, whose motility is markedly enhanced and associated with high tumor-initiating and metastatic capacity.

In order to verify whether a gradient of CXCL11 and CXCL12 could enhance meningioma cell migration, directional migration towards a chemotactic stimulus (CXCL11 or CXCL12) was assessed in 8 fluorescent-labelled meningioma cultures by trans-well assay, as reported in Figure 27A.

Chemotactic migration was quantified by counting the number of migrated cells from images of cells on the bottom well membrane captured after o/n incubation (Figure 27A-B).

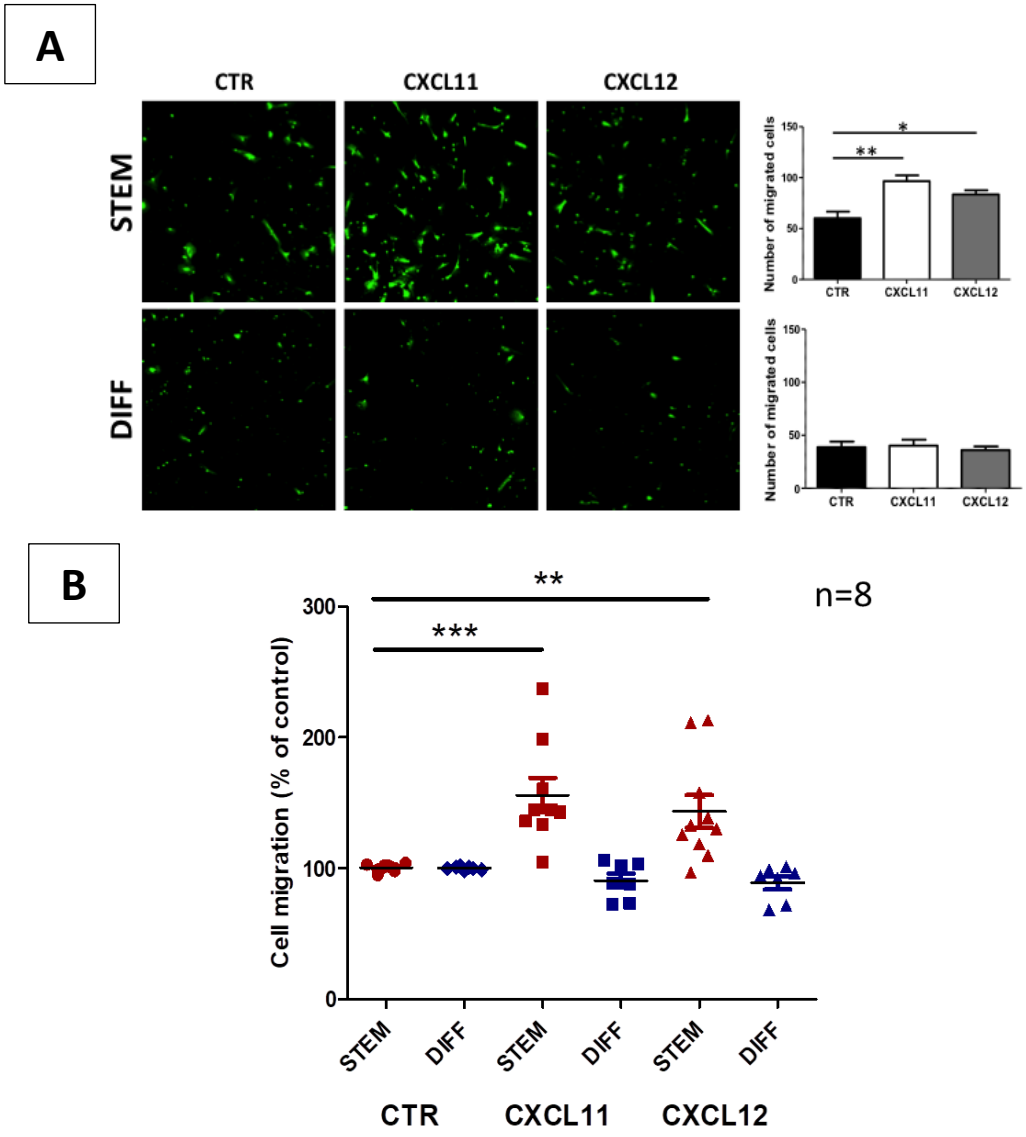

Figure 27 Effects of CXCL11 and CXCL12 on the migratory potential of STEM and DIFF cells.
A. Representative migration pictures of the bottom side of the membrane at the 15h time-point, and histograms of quantification of STEM and DIFF cultures plated in the top chamber of a transwell plate, and CXCL11 (25 nM) or CXCL12 (25 nM) faded to the bottom chamber from meningioma M1-17. **B.** Histogram represents the mean ± s.e.m. of chemotaxis quantification from 8 cultures (***p<0.001, **P<0.01; *vs.* respective CTR, t-test).

The migratory response of STEM cells to CXCL11 and CXCL12 was significantly higher as compared with untreated controls, highlighting the stem-like features of these cultures. Otherwise, no cell migration was observed in response to both CXCL11 and CXCL12 in DIFF cultures. The lack of cell migration response towards chemotactic ligands in DIFF cultures confirms the results obtained by proliferation assay.

Meningioma, commonly does not metastasize outside the CNS, however, brain invasion is frequent and represents a key clinical parameter included among the criteria used to classify grade II tumors in the last WHO classification and, more in general to identify an aggressive behavior of meningiomas, considering that a percentage of benign tumors behaves atypically as far as prognosis due to brain invasive ability.

CXCL12/CXCR4 axis controls cell migration and chemotaxis, and, likely, in STEM meningioma cell this chemotactic behavior does not only affect the homing of CSC-like subpopulation within the tumor niche, but also contributes to infiltration of the adjacent brain tissues. Histopathological studies in meningiomas describe either groups of tumor cells invading brain parenchyma or single cells spreading into neural tissue regardless of tumor location [247]. The process by which meningioma cells spread out the primary tumor represents a major determinant of poor prognosis favoring tumor recurrence due to incomplete surgery for the presence of cluster of cells dispersed in the brain [248]. The highly migratory nature observed in STEM meningioma cultures further support their stem-like nature, an extremely interesting feature taking into account the histopathological benign origin of these cells and outlining the partially inconsistent biologic value of histopathologic grading in meningiomas. In addition, CXCL11- and CXCl12-dependent migration of meningioma stem-like cell likely arise in vivo as a consequence of a chemokine gradient which act as chemoattractant for tumor cell expressing specific receptors, creating directional migration within brain parenchyma. As observed in other solid tumors, CSC-like subpopulation in meningioma may be characterized by higher

plasticity and inherent migratory potential than more differentiated cells favoring their robust response to chemoattractants in tumor microenvironment.

3.8 CXCL11 and CXCL12 promote tube formation in STEM cultures

Given the high vascularization which characterizes meningiomas, and the pro-angiogenic activity of CXCR4 and CXCR7 in several tumors, we completed the functional study evaluating the capacity of CXCL11-CXCL12/CXCR4-CXCR7 interactions to stimulate *in vitro* formation of vascular structures. Tube formation assay was performed in 8 meningioma STEM and DIFF cultures. In order to induce tube formation, cells were shifted from their respective culture media in Basal EndoGro medium (in the Figure 28, named ENDO-BASAL), and after 24h cells were plated on Matrigel-coated slides in the presence of 25 nM CXCL11 or CXCL12. The formation of vessel-like structures has been monitored after 6h by phase-contrast microscopy. Under basal condition STEM cells were already able to form tubes than DIFF cells although slightly, whereas STEM cells displayed higher capacity to transdifferentiate into endothelial-like cells, giving rise to a considerably larger number of loops than DIFF cells, after exposure to CXCL11 and CXCL12 stimuli.

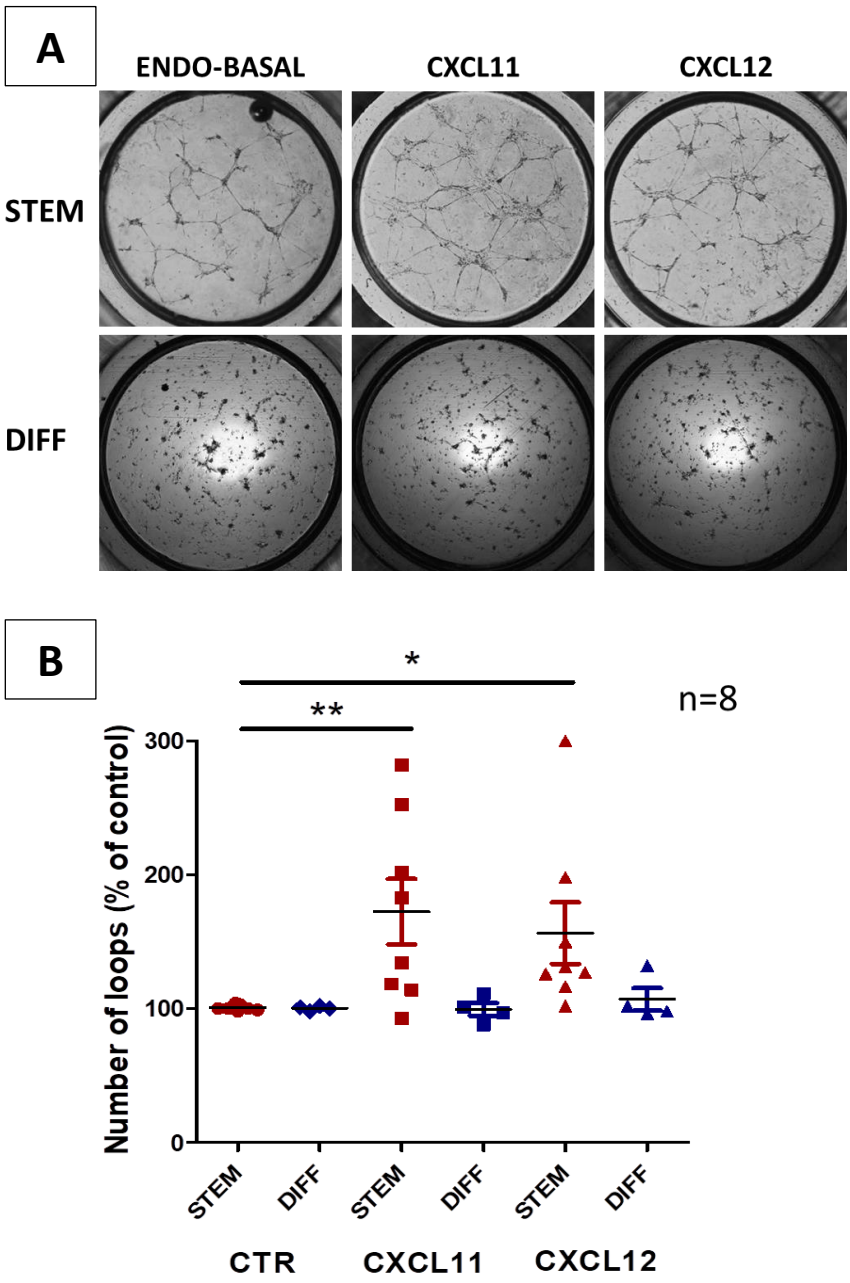

Figure 28 Effects of CXCL11 and CXCL12 on formation of vascular structures of STEM and DIFF cells. **A.** Representative pictures of STEM and DIFF meningioma M1-17 cells, stimulated with CXCL11 (25 nM) or CXCL12 (25 nM). **B.** Angiogenesis from 8 cultures was quantified by mean count of loop number ± S.D. (*p<0.05, **p<0.01, *vs.* respective CTR, t-test).

Overall, these results highlight two main observation: first of all, that chemokines, and CXCL12 and CXCL11 in particular, are main regulators of relevant biological activities likely involved in the aggressive behavior of meningiomas (i.e. proliferation, migration and neo-angiogenesis), but that, independently on the expression levels of the relative receptors (CXCR4 and CXCR7), different

subpopulations of cells display a selective ability to respond to the chemokines. In particular, being higher proliferative, migratory, and tube formation activities were observed in STEM cultures and almost absent in DIFF cultures, we hypothesized that the atypical clinical course of some grade I meningioma might by related to a larger presence of this subpopulation. Thus, if these results will be validated in larger series of meningiomas, their histological identification mat represent a novel prognostic index for those with unpredictable clinical course.

3.9 Pharmacological inhibition of both CXCR4 and CXCR7 inhibits CXCL11- and CXCL12-induced proliferation, tube formation and migration of meningioma STEM cells

On the basis of the ability of CXCL11 and CXCL12 to trigger proliferative, migratory and angiogenic responses, selectively observed in STEM culture subpopulation, we decided to delve deeper into the individual contribution of receptors for these chemokines. CXCR4 and CXCR7 role in meningioma STEM cell functioning was pursued by pharmacological inhibition of this chemokine system using antagonists for CXCR4 (AMD3100) and CXCR7 (CCX771). However, the pharmacology of these receptors is rather complex, since CXCR4 is bound only by CXCL12 and antagonized by AMD3100, but CXCR7 is bound by both CXCL12 and CXCL11 (which also activates CXCR3) and is blocked by CCX771 (although a single report suggested that AMD3100 could act as partial agonist for CXCR7 [231], but no subsequent studies confirmed this evidence). Notwithstanding, the combination of the aforementioned agonists

and antagonists is to date the best pharmacological approach to define the biological roles of these receptors.

Thus, 24h-starved STEM cells, derived from 6 meningiomas, were incubated with CXCL11 (25 nM) or CXCL12 (25 nM), in the presence or absence of AMD3100 (1µM) and CCX771 (10 nM) for 24h before performing MTT assay (Figure 29).

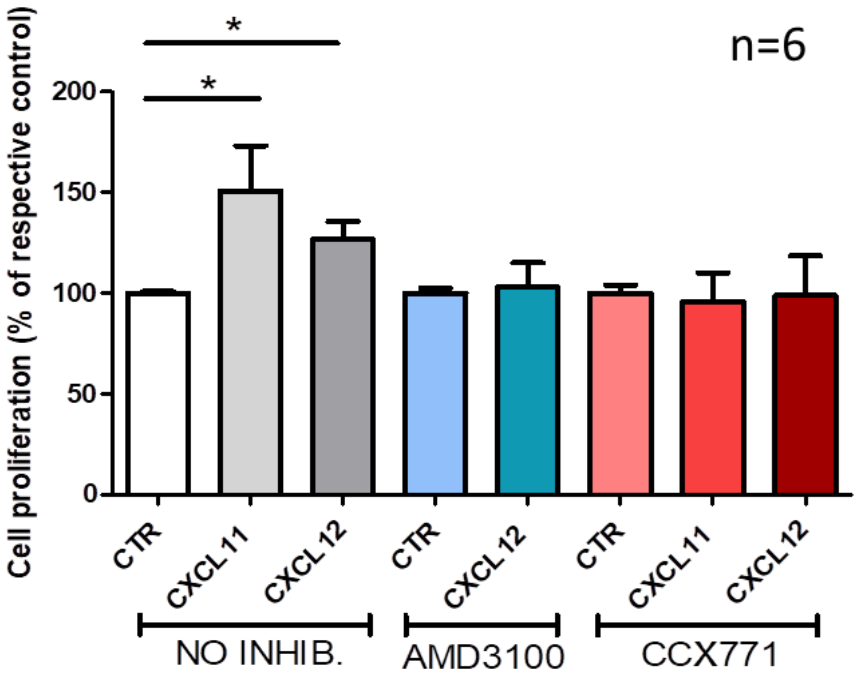

Figure 29 Proliferation on six STEM cultures stimulated with 25 nM CXCL11 or 25 nM CXCL12 and treated with the inhibitors of CXCR4 (AMD3100, 1 µM) or CXCR7 (CCX771, 10 nM) by MTT assay (*p<0.05, *vs.* respective CTR, t-test).

The ability of both chemokines to significantly increase STEM cell proliferation was significantly hampered in the presence of the antagonists. Treatment of cultures with the CXCR4 antagonist AMD3100 prevented CXCL12-induced proliferation, while CCX711 reduced the stimulation of both CXCL11 and CXCL12. Since the effect of both ligands were blocked by both antagonists, our data allows us to hypothesize that the proliferative effect of chemokines is mediated by both CXCR4 and CXCR7,

and that, as far as CXCL12, the single inhibition of each of its receptors is enough to prevent the activation of proliferation. In this context, it has to be remarked that CXCR4 and CXCR7 have been reported to dimerize in the presence of ligand activation, thus it is likely that the antagonists exert their action preventing CXCL12-dependent dimer formation or blocking the signaling of preformed dimers. Further studies will be necessary to address this issue.

In parallel, we evaluated, by Western Blot, CXCL11- or CXCL12-dependent ERK1/2 phosphorylation/activation in 4 STEM meningioma cultures, after pre-treatment with AMD3100 (1μM) or CCX771 (10nM) (Figure 30). CXCL12 (25nM) induced a statistically significant ERK1/2 activation in STEM meningioma cells, while CXCL11 stimulation of ERK1/2 phosphorylation, although clearly evident in few samples, on the average did not reach a statistical significance.

However, pre-treatments with AMD3100 and CCX771 completely prevented CXCL12- and CXCL11-activation of ERK1/2 (Figure 30).

Overall, above results suggest that survival/proliferation of meningioma STEM cell culture caused by chemokine stimulation of CXCR4 and CXCR7 is mediated by pathways that requires signaling by ERK1/2.

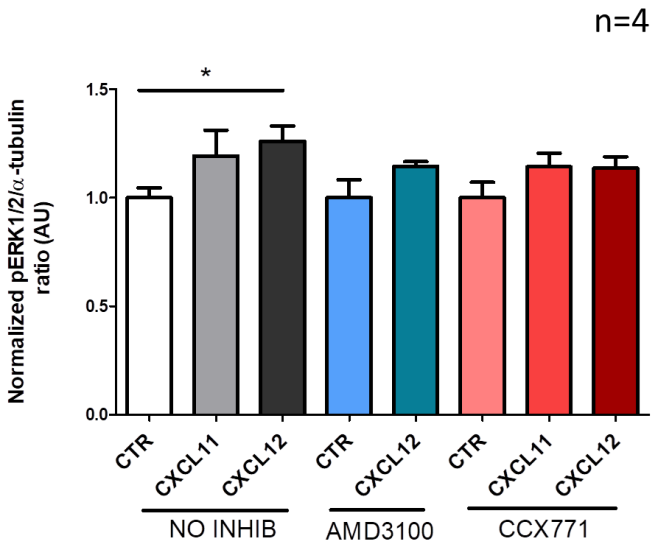

Figure 30 Quantification of p-ERK1/2 level by densitometric analysis as mean ± S.D., collecting data from 4 meningioma STEM cultures (*p<0.05, *vs*. respective CTR, t-test).

As previously observed in the Figure 26, in which six different meningiomas were analyzed, the effect of the stimulation of CXCL11 is not significant since of the four meningiomas investigated, two not respond with the activation of ERK1/2. Although average, an increase in phospo-ERK1/2 is observed.

Next, we investigated the impact of the blockade of CXCR4 and CXCR7 signaling, using AMD3100 and CXC771 on cell migration (Figure 31). The significant CXCL11 and CXCL12-induced chemotaxis (Figure 31) is reverted to basal levels by both pharmacological antagonists, thus indicating that the migration depedent by these cxhemokine involves both receptors, and suggesting that blocking CXCR4-7 signaling may be a potential therapeutic approach to inhibit highly invasive meningioma cells.

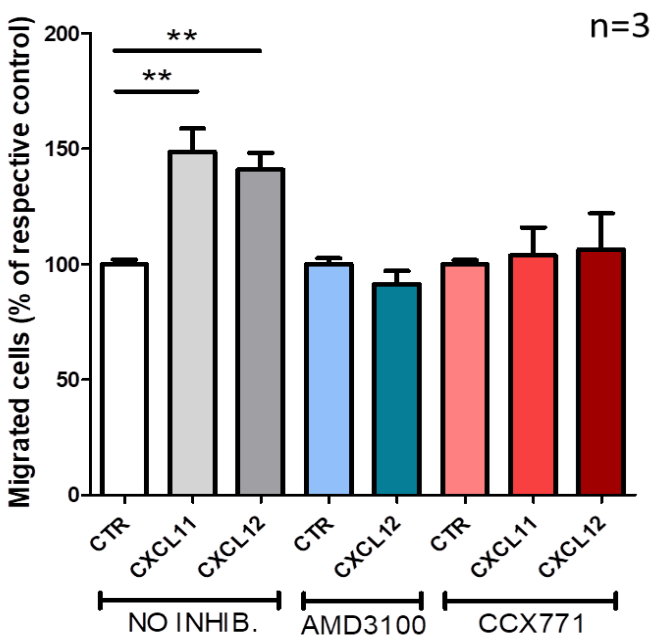

Figure 31 Migration on 3 STEM cultures stimulated with 25 nM CXCL11 or 25 nM CXCL12 and treated with the inhibitors of CXCR4 (AMD3100, 1 µM) or CXCR7 (CCX771, 10 nM) (*p<0.05, *vs*. respective CTR, t-test).

These results are particularly relevant as far as the role of CXCR7. Indeed, CXCL12-CXCR4 signaling has been previously implicated in migration and invasiveness of several tumor types, and, in particular, in the homing of cancer cells to the metastatic site; conversely, CXCL12 signaling in the migratory potential of tumor cells expressing CXCR7 and the mechanisms by which CXCR7-mediates invasion has been scantly investigated in most tumors including meningioma.

We also analyzed the ability of pharmacologic inhibition of CXCR4 and CXCR7 to modulate chemokine-enhanced angiogenesis *in vitro* (Figure 32). AMD3100 and CCX771 treatment markedly reduced the tube formation induced by both CXCL11 and CXCL12 in STEM meningioma cultures, bringing back the vessel-like structure formation to control levels. It is well known that CXCL12 regulates angiogenesis, mainly through its receptor CXCR4; while, also in this case, the role of CXCR7 in

angiogenesis is mostly reported in endothelial cell, being this receptor highly expressed in blood vessel of a variety of tumors. On the other hand, CXCR7 function in cancer stem cells is less definite. Taken together our results on the CXCL12-CXCR7 interaction involved and likely sustaining stem phenotype of STEM meningioma is relevant in this high vascularized tumor.

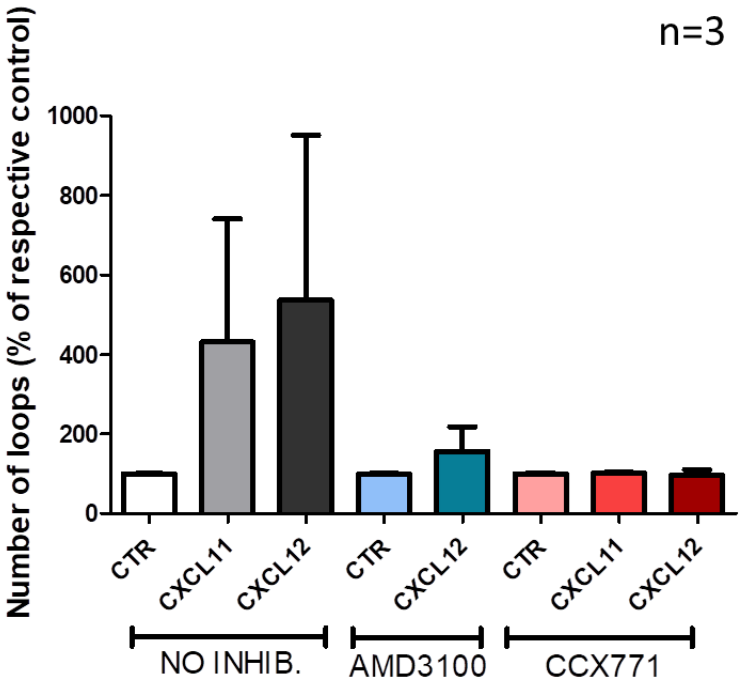

Figure 32 Tube formation assay on 3 STEM cultures stimulated with 25 nM CXCL11 or 25 nM CXCL12 and treated with the inhibitors of CXCR4 (AMD3100, 1 µM) or CXCR7 (CCX771, 10 nM) (*p<0.05, *vs.* respective CTR, t-test).

4. Conclusions

Cancer stem cell (CSC) hypothesis propose that, within tumor cell heterogeneity, a small subpopulation of cells, named CSCs, is responsible for tumor formation, growth, spreading, and recurrence. Generally, CSC concept is applied to malignant tumors, while less is known about their role in benign lesions such as the majority of meningiomas. Nevertheless, the definition of CSC phenotype is challenging due to technical limitations, lack of univocal marker definition, different functional assays and heterogeneity of cellular hierarchy within both benign and malignant tumors.

In this context, one main issue addressed in the research reported in this thesis has been the identification, isolation, and *in vitro* expansion of putative meningioma stem-like cells, from a large series of unselected human post-surgical meningioma. Moreover, this cell subpopulation has been challenged for multiple functional assays related to the known CSC activities.

Our results highlight that different cell subpopulations are present within meningiomas, including stem-like cells which are able to survive and grow in stem cell-permissive conditions *in vitro*; these cells show remarkable distinctive features as compared to their "non-stem" counterpart: 1) higher proliferation rate; 2) ability to self-renew; 3) expression of several stem markers; 4) multilineage differentiation ability (although in this case only few STEM cultures show a mesenchymal-like differentiation ability); 5) increased migratory capacity; 6) capability to undergo the formation of tridimensional tube formation (vascular mimicry). Further studies are

currently in progress to demonstrate the *in vivo* tumorigenic activity of these cells, which represent the definitive proof of the CSC nature of these cells.

As expected from meningioma histopathologic distribution and incidence, the great majority of the tumors entered in the study were grade I, morphologically benign lesions, but seldom exhibiting an aggressive clinical behavior, relapsing and undergoing malignant transformation. Grading and altered molecular pathways of meningiomas are often identified based on the bulk of tumor cells, therefore the biological features of meningioma stem cell-like subpopulation we isolated might help to obtain key information on their distinct role in meningioma aggressiveness, currently scarcely investigated. Moreover, this study was performed on early passage cell cultures, after the isolation from fresh surgical tissue, thus strictly reflecting the patients' tumor landscape rather than obtainable using cell lines, is also relevant in the perspective of pharmacological studies. Indeed, pharmacological targeting of CSCs could represent a relevant and still unidentified novel strategy, in particular for recurrent, progressive and symptomatic meningiomas, for which to date there are no effective therapies. In this view, a future development of this work should be the assessment of the tumorigenic activity of meningioma stem-like subpopulation to obtain a better pre-clinical model to be exploited for both molecular and therapeutic studies. This study also contributes to improve the knowledge of mesenchymal cell markers (CD105, CD90 and CD73) which have been proposed to identify putative meningioma stem cells, particularly CD105. In our series of meningioma cultures, CD105, was similarly expressed in both stem and "non-stem" cultures which also co-expressed CD90 and CD73. Therefore, we propose that CD105 is not a selective stem marker for

meningioma CSCs, and likely other mesenchymal stem cell surface proteins might play additional role in meningioma cells. Beside tumor heterogeneity, recent oncology studies attempted to include CSCs within tumor microenvironment and stem cell-like niche, to delve deeply in the mutual interactions between these structures that lead to tumor complexity. The communication between cells and tumor microenvironment, is critical for tumor growth, angiogenesis, and metastasis, all representing hallmarks of malignant transformation. In particular, CXCR4/CXCR7-CXCL12/CXCL11 axis sustains proliferation, self-renewal, migration and invasion of CSCs in different cancers, while little is known in meningioma model. In this context, we analyzed the expression of the chemokines CXCL11-CXCL12 and their receptors CXCR4-7 in meningioma stem-like cells and "non-stem" cells, observing an inverse correlation between the expression level of ligands and their receptors. These results suggest that the upregulation of the ligands in subsets of cells might induce a chronic activation of the respective receptors in a paracrine/autocrine manner causing a phenotypical reduced expression of the receptor, and *vice versa*. Both chemokines selectively promoted *in vitro* proliferation, migration and tube formation of meningioma stem-like subpopulation, while "non-stem" cells did not respond to the chemokinergic stimuli. Overall, these results suggest the CXCL11/CXCL12-CXCR4/CXCR7 system plays a relevant role in meningioma stem cell maintenance and underline an individual role for CXCR4 and CXCR7 as pro-angiogenic and pro-migratory mediator of the high vascularization and invasive features of aggressive meningioma. Our data, demonstrating a sustained CXCL12-CXCR7 interaction in stem cell phenotype and functions is relevant since CXCR7 activity in CSCs is less known and definite than CXCR4-dependent mechanisms,

adding further components in the CSC-microenvironment cell crosstalk. These data further suggest that stem cell subset do not passively reside within the tumor mass but also regulate the cell functioning by producing soluble factors such as chemokines. We speculate that the malignant phenotype of some grade I meningioma might by related to a larger presence of this subpopulation. CXCR4-7 signaling could also represent a future clinical target, since effective therapeutic approaches antagonizing the activity of this receptor likely might also affect CSC-tumor microenvironment crosstalk. Finally, we demonstrated the ability of CXCR4 and CXCR7 antagonists (AMD3100 and CCX771, respectively), individually added, to completely prevent meningioma stem-like cell proliferation, migration and neo-angiogenesis induced by both CXCL11 and CXCL12. These data indicate that meningioma stem cells functions are mediated by both CXCR4 and CXCR7, possibly acting as heterodimers, since the single inhibition of each of its receptor is sufficient to block the activity of both receptors. Formation of CXCR4-CXCR7 heterodimers, in the presence of ligand activation, seems to be crucial in the highly aggressive response of CSCs in several tumors. In meningioma cells, the antagonists exert their action preventing CXCL12-dependent dimer formation or blocking the signaling of preformed dimers; further studies will be necessary to address the inhibition of the chemokine pathway as a possible successful strategy against recurrent meningioma. In conclusions, we demonstrate that also in meningioma is present a cellular subpopulation endowed with several phenotypical and biological features of CSCs, that being under control of the CXCL12/CXCL11 chemokinergic system, might be pharmacologically targeted by selective antagonists of their receptors.

5. Materials and Methods

5.1 Isolation and culture of human meningioma cells

All meningioma samples were collected from the Neurosurgery Unit of the Ospedale Policlinico San Martino – IRCCS of Genova, after Ethical Committee approval and informed consent obtained from all patients.

Thirty-seven surgical meningioma specimens were processed upon arrival in the laboratory, for single cell dispersion by mechanical disaggregation with sterile forceps and scalpels, and then filtered through a 70 µm strainer to remove possible impurities and aggregates.

Cell suspensions were collected and were cultured in DMEM/F12 (1:1), supplemented with 1% penicillin-streptomycin (Lonza) and 2 mM L-glutamine (Lonza) containing 10% fetal bovine serum (FBS, Lonza) for a week.

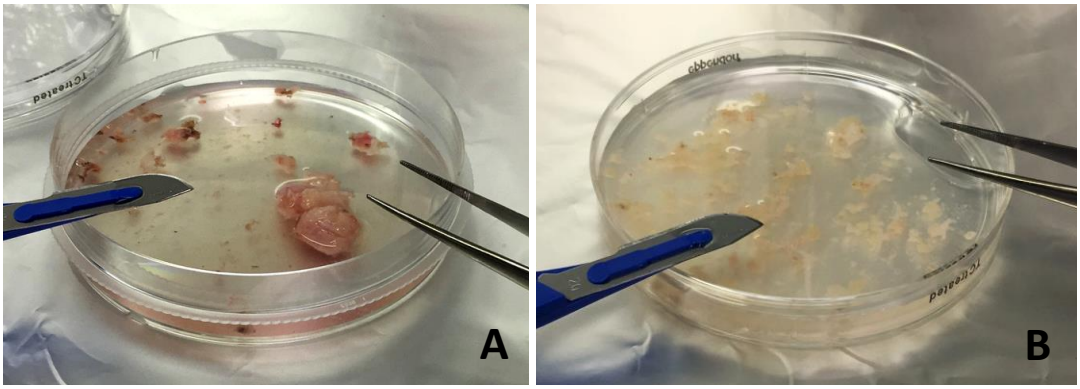

Figure 33 Meningioma specimen derived from surgical resection (A); single cell dispersion by mechanical disaggregation of the tumor mass (B).

After 1 week, primary cultures able to grow as adherent cells, were harvested by enzymatic mean (Trypsin) to remove possible contaminating cells sub-cultured in two distinct media:

1. **STEM**-culture in stem cell-permissive medium: DMEM-F12/Neurobasal (1:1) supplemented with 1% B27 (Life Technologies), 2 mM L-glutamine (Lonza), 1% penicillin-streptomycin (Lonza), 10 ng/ml bFGF and 20 ng/ml EGF (Milteny Biotec);
2. **DIFF**-culture in DMEM/F12 medium containing FBS 10%: DMEM/F12 (1:1) supplemented with 1% penicillin-streptomycin (Lonza) and 2 mM L-glutamine (Lonza), 10% FBS (Lonza).

To obtain more experimentally manageable cultures, cells maintained in stem-permissive medium were grown in dishes/flasks coated with Matrigel (BD Biosciences) as monolayer.

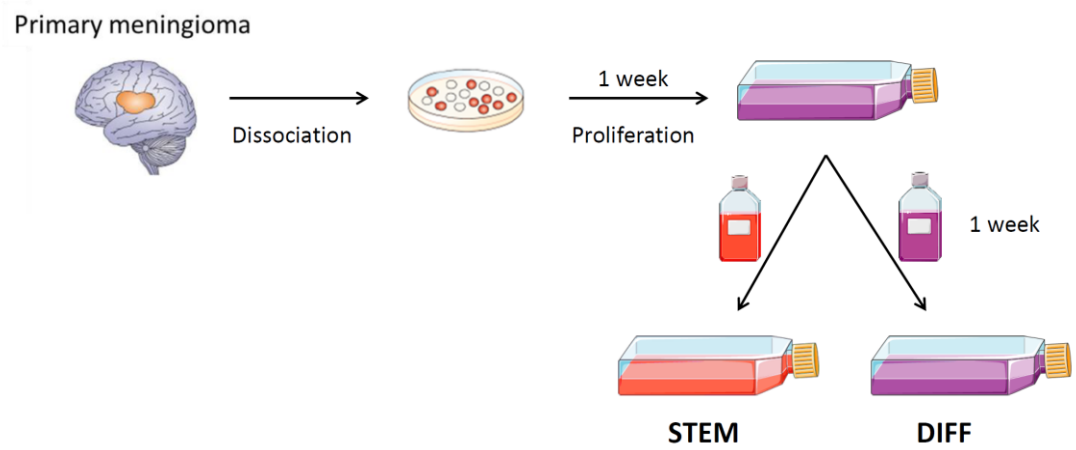

Figure 34 Isolation and growth of meningioma STEM and DIFF cells.

5.2 Fluorescence Activated Cell Sorting (FACS)

FACS analysis was performed to investigate the surface antigen expression profile of cells obtained from tissue disaggregation at passage 0, and of one week after splitting-cells in the two different culture conditions (p1).

Cells were washed with 1 ml of FACS buffer (phosphate buffered saline, PBS/ EDTA 2 mM, 0.5% bovine serum albumin BSA, 0.5%) and resuspendend with phycoerythrin-fluorescein isothiocyanate (FITC)-, Alexa Fluor 647-, or allophycocyanin-conjugated antibodies against CD105 (Miltenyi Biotec), CD31 (Miltenyi Biotec), CD106 (Miltenyi Biotec), CD14 (BD Biosciences), HLA-DR (BD Biosciences), CD45 (BD Biosciences), CD34 (BD Biosciences) and CD19 (BD Biosciences). All antibody solutions were prepared in FACS buffer. The dead cells were excluded from analysis by adding 7-aminoactinomycin-D (7-AAD) (BD Biosciences). Data were acquired on BD FACSCanto II (BD Biosciences) and analyzed by BD FACSDiva software.

With same preparation, cells were stained for CD105, CD90 and CD73 by Human MSC Phenotyping kit (Miltenyi Biotec), and resuspend in LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit in accordance with manufacturer's instructions (Molecular Probes, Life Technologies) Appropriate IgG isotype-matched antibodies and unstained cells were used as controls. Data were acquired on BD FACSCanto II (BD Biosciences) and analyzed by BD FACSDiva software.

5.3 Cell proliferation assay

Mitochondrial activity, as index of cell viability, was evaluated by measuring the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich).

Cells (5,000/well) were seeded in 48-well plates to allow exponential cell growth for duration of the assay (up to 7 days). Cells were incubated with MTT 0.25 mg/ml for 2h, formazan crystals dissolved in 100 µl DMSO and absorbance measured at 570 nm.

MTT assay was also performed in 96-well plates containing 1,500/well and treated with chemokines (CXCL11 25 nM or CXCL12 25 nM), and in the presence of absence of the respective inhibitor: AMD3100 (1 µM) and CCX771 (10 nM) for 24h.

5.4 Meningosphere-formation assay

Cells deriving from both STEM and DIFF cultures were collected and resuspended in STEM medium, seeded in 6-well ultra-low attachment plates (Corning) at 50,000 cells/well. Sphere formation was monitored for up to 7 days and cultures were analyzed by light microscopy; images of each individual well were acquired with a digital camera Leica ICC50 HD (Leica) mounted on a transmitted light microscope DM IL (Leica).

5.5 Immunofluorescence (IF)

Cells (10,000/well) were plated on 8-well chamber slide (BD Bioscience), previously -coated with Matrigel for 30 min (STEM cells) or without coating (DIFF cells). After 24 h, cells were fixed with 4% paraformaldehyde (PFA) in PBS for 10 min, washed twice with PBS, permeabilized with PBS/0.1% Triton X-100, blocked with normal goat serum (NGS) (all from Sigma-Aldrich) and incubated with the following primary antibodies: Sox2 (mouse monoclonal, Abcam), Oct4 (rabbit polyclonal, Abcam), Nanog (rabbit polyclonal, Abcam), CD133 (mouse monoclonal, Novus Bio), CXCR4 (mouse monoclonal, Abcam), CXCR7 (rabbit polyclonal, Abcam), CXCL11 (rabbit polyclonal, Abcam), CXCL12 (rabbit monoclonal, Abcam), E-cadherin (mouse monoclonal, Abcam), N-cadherin (rabbit polyclonal, Abcam), all diluted 1:100, and CD105 (mouse monoclonal, Abcam) diluted 1:200, in 2% NGS-PBS at r.t for 1 h. Fluorochrome-conjugated antibodies (goat anti-mouse and anti-rabbit Alexa Fluor-568 or Alexa Fluor-488, Molecular Probes-Invitrogen) were applied, and nuclei were counterstained with DAPI (Sigma-Aldrich). Negative controls omitting primary antibodies were included in all the experiments. Coverslips were mounted with ProLong™ Gold Antifade Mountant (ThermoFisher). Slides were photographed with DM2500 microscope (Leica) equipped with DFC350FX digital camera (Leica).

5.6 Western Blot

Cultures were washed and lysed in a buffer containing 20 mM Tris-HCl pH 7.4, 140 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Igepal, 1mM sodium orthovanadate, 1mM

phenylmethyl-sulfonyl fluoride, 10mM NaF (all from Sigma-Aldrich), and the "Complete" protease inhibitor mixture (Roche Applied Science), for 10 minutes at 4°C. Nuclei were removed by centrifugation (5,000 rpm at 4°C) for 10 min and total proteins were quantified using Bradford assay (Bio-Rad).

Proteins (20-60 µg) were resuspended in Laemmli buffer (2% SDS, 62.5mM Tris, pH 6.8, 0.01% bromphenol blue, 1.43 mM b-mercaptoethanol, 0.1% glycerol), subjected to 10% SDS-PAGE, electroblotted onto polyvinylidene of fluoride membrane (Bio-Rad) and probed with specific antibodies directed against CD105 (endoglin, rabbit polyclonal, Santa Cruz), Sox2 (mouse monoclonal, Abcam), and beta-actin (mouse monoclonal, Cell signaling).

Immunocomplexes were detected using a chemiluminescence system (BioRad Laboratories), and quantified by the ChemiDoc XRS apparatus (Bio-Rad Laboratories).

When convenient, STEM culture and DIFF, culture media were deprived of growth factors and FBS, respectively, for 24 h before CXCL11 (25 nM) and CXCL12 (25 nM) stimuli, to remove possible interference. Thus, cells were treated for 5 min, in the presence or absence of the respective inhibitor: AMD3100 (1 µM) and CCX771 (10 nM). As above, 40 µg of proteins were probed with specific antibodies direct against phospho-ERK1/2 (Cell Signaling Technology) and alpha-tubulin (mouse monoclonal, Cell Signaling).

5.7 Multilineage differentiation analysis

The ability to differentiate into mesenchymal lineages (adipogenic, chondrogenic and osteogenic lineages) of putative meningioma CSCs was verified by switching the cultures into specific-enriched factors medium. Briefly, 15,000 cells were seeded and cultured in StemPro® Osteogenesis Differentiation medium (GIBCO®) replacing medium every 3 days, and after 10 days stained with 2% alizarin red S (Sigma-Aldrich) to detect the formation of calcium deposits. Otherwise, adipogenesis differentiation ability was evaluated by seeding 25,000 cells in StemPro® Adipogenesis Differentiation medium (GIBCO®) for 21 days and replacing medium every 3 days. The presence of intracellular lipid vacuoles typical of adipocytes, was detected by using Oil Red O (Sigma-Aldrich) staining. Chondrogenic differentiation was assessed by plating 40,000 cells/well in NH ChondroDiff Medium (Miltenyi Biotec GmbH, Germany) for 21 days, replacing medium every 3 days. Chondrogenesis was confirmed using Alcian Blue staining (Sigma-Aldrich).

Images were acquired with a digital camera Leica ICC50 HD (Leica) mounted on a transmitted light microscope DM IL (Leica).

5.8 Cell migration assay

STEM and DIFF cultures were labeled with the fluorescent dye Vybrant® CFDA SE Cell Tracer Kit (following the manufacturer's instructions, for 15 min at 37°C. After 30 min in DMEM at 37°C, in which CFDA will undergo acetate hydrolysis, cells were suspended and counted. Migration was performed in FluoroBlok™ HTS 96 multiwell

culture inserts (8 µm pore size, Corning) by seeding 15,000 cells to the upper chamber in 50 µL DMEM, and 225 µL DMEM supplemented with CXCL11 (25 nM) or CXCL12 (25 nM) or 10% FBS were placed in the lower chamber [249]. Each condition was performed in triplicate. After overnight incubation under 5% CO_2 at 37°C, three microscopic fields of the lower surface of the filter in each well were acquired with a confocal laser scanning microscope (BioRad MRC 1024 ES). Intensity quantification of fluorescence of migrated cells was counted using "Analyze Particles" tool by ImageJ software (NIH, Bethesda, USA).

5.9 Tube formation assay

µ–Slide Angiogenesis (Ibidi) were coated with 10 µL Matrigel (BD Biosciences) and allowed to polymerize at 37°C for 30 min. After 24 h of starvation in EndoGro™ basal medium (Merck Millipore), 10,000 cells from both STEM and DIFF cultures, were seeded on Matrigel-coated wells and incubated at 37°C in EndoGro™ basal medium and EndoGRO™-VEGF complete medium (Merck Millipore) [250] for 6h to allow tube formation. Tube formation assay was also performed on cells treated with chemokines (CXCL11 25 nM or CXCL12 25 nM), and in the presence of absence of the respective inhibitor: AMD3100 (1 µM) and CCX771 (10 nM) for 6h.

Tube formation in each well was photographed using a phase contrast microscope DM IL (Leica) and pictures were acquired with a digital camera Leica ICC50 HD (Leica).

To quantify the results, the mean of loops, in which at least 3 tubes joined, were counted using the ImageJ software.

5.10 RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA has been extracted using the High Pure RNA Isolation Kit (Roche Applied Science, Manheim, Germany), according to the manufacturer's instruction and it has been retro-transcribed into cDNA using the iScript cDNA Synthesis Kit (BioRad Laboratories). Single stranded cDNA products were analyzed by real-time PCR using the SsoFastTM EvaGreen mix (BioRad Laboratories) on a CFX96 Touch real-time PCR (BioRad Laboratories). Cycling conditions were set at 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds, for 35 cycles.

Primer sequences have been designed on the mature transcript:

- **CXCL12:** forward 5' - GCCCGTCAGCCTGAGCTACA- 3'

  reverse 5' - TTCTTCAGCCGGGCTACAATCT - 3'
- **CXCL11:** forward 5' - GAGTGTGAAGGGCATGGCTA - 3'

  reverse 5' - TAAGCCTTGCTTGCTTCGAT - 3'
- **CXCR4**: forward 5' - TTCTTCAGCCGGGCTACAATCT - 3'

  reverse 5' - GGAACACAACCACCCACAAGT -3'
- **CXCR7**: forward 5' - CAACCCTGTCCTCTACAGCTTCAT – 3’

  reverse 5' - TCATTTGGTGCTCTGCTCCAAG - 3'
- **VEGFR2**: forward 5’ – CACCACTCAAACGCTGACATGTA – 3’

  reverse 5’ – GCTCGTTGGCGCACTCTT – 3’

Human β-actin and 28s pre-designed PrimePCR pair primers (Bio-Rad) have been used as internal controls. Levels of target genes in each sample were normalized on the basis of housekeeping gene amplification and reported as relative values. All

qRT-PCR runs included negative controls without mRNA templates and cDNA transcription to check reagents for contaminations; moreover, melting- curve has been performed at the end of each amplification-run set in order to evaluated the specificity of the reaction.

5.11 Statistical analysis

Statistical significance between groups was assessed by t-test (unpaired, two-tailed).

Statistics were performed using Prism version 5.02 (GraphPad San Diego CA, USA). Statistical significance was established at p values <0.05.

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250. Corsaro, A., et al., *Cellular prion protein controls stem cell-like properties of human glioblastoma tumor-initiating cells.* Oncotarget, 2016. **7**(25): p. 38638-38657.

List of Publications

1. Barbieri F, Würth R, Pattarozzi A, Verduci I, Mazzola C, Cattaneo MG, Tonelli M, **Solari A**, Bajetto A, Daga A, Vicentini LM, Mazzanti M, Florio T.
Inhibition of Chloride Intracellular Channel 1 (CLIC1) as Biguanide Class-Effect to Impair Human Glioblastoma Stem Cell Viability.
Frontiers in Pharmacology 2018; 9:899.
I.F: 3,831

2. Baldassari S*, **Solari A***, Zuccari G, Drava G, Pastorino S, Fucile C, Marini V, Daga A, Pattarozzi A, Ratto A, Ferrari A, Mattioli F, Barbieri F, Caviglioli G, Florio T.
Development of an Injectable Slow-Release Metformin Formulation and Evaluation of Its Potential Antitumor Effects.
Scientific Reports 2018; 8(1):3929.
* Equally contributed as first author
I.F: 4,122

3. Gatti M*, **Solari A***, Pattarozzi A, Campanella C, Thellung S, Maniscalco L, De Maria R, Würth R, Corsaro A, Bajetto A, Ratto A, Ferrari A, Daga A, Barbieri F, Florio T.
In vitro and in vivo characterization of stem-like cells from canine osteosarcoma and assessment of drug sensitivity.
Experimental Cell Research 2018; 363(1):48-64.
* Equally contributed as first author
I.F: 3,309

4. Angeletti F, Fossati G, Pattarozzi A, Würth R, **Solari A**, Daga A, Masiello I, Barbieri F, Florio T, Comincini S.
Inhibition of the Autophagy Pathway Synergistically Potentiates the Cytotoxic Activity of Givinostat (ITF2357) on Human Glioblastoma Cancer Stem Cells.

Frontiers in Molecular Neuroscience 2016; 9:107.

I.F: 5,076

5. Corsaro A, Bajetto A, Thellung S, Begani G, Villa V, Nizzari M, Pattarozzi A, **Solari A**, Gatti M, Pagano A, Würth R, Daga A, Barbieri F, Florio T.

Cellular prion protein controls stem cell-like properties of human glioblastoma tumor-initiating cells.

Oncotarget, 2016; 7(25):38638-38657.

I.F: 5,008

List of Abstracts

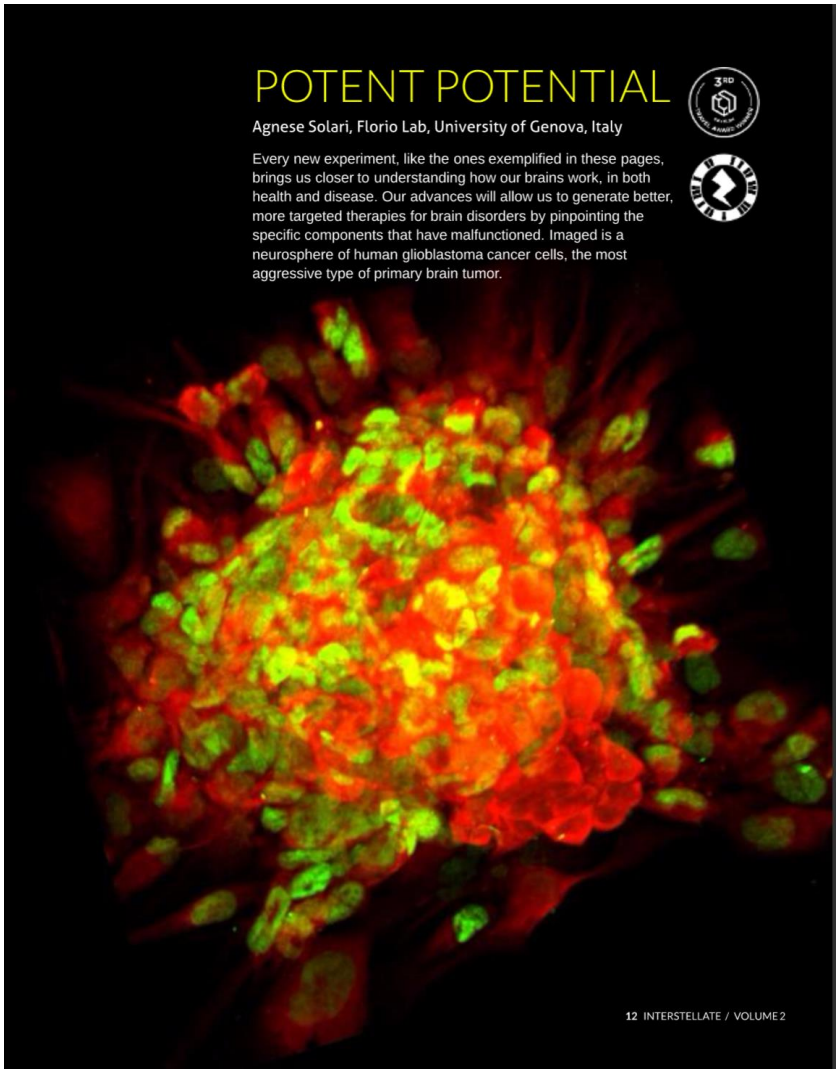
1. Florio T., Barbieri F., Wurth R., Verduci I., Cattaneo M., **Solari A**., Daga A., Vicentini L. M., Mazzanti M. "Different biguanide-related drugs eradicate human glioblastoma stem cell through the inhibition of chloride intracellular channel 1 activity", Neuroscience 2018 (San Diego, CA, USA) 3-7 Nov, Abs 659.07 / V1, (2018).
2. **Solari A.**, Rogna L., Fiaschi P., Zona G., Barbieri F., Florio T. "Role of CXCR4/CXCR7 - CXCL11/CXCL12 axis in putative stem cells from human meningioma", poster presentation at "Cambridge International Stem Cell Symposium", Cambridge, UK, 19-21 September, 2018.
3. **Solari A.**, Rogna L., Fiaschi P., Zona G., Barbieri F., Florio T. "Role of CXCR4/CXCR7 - CXCL11/CXCL12 axis in putative stem cells from human meningioma", BYNS 2018 "II Barcelona Young Neuroscientists Symposium ", Barcelona, Spain, 13-14 September, 2018.
4. Rogna L., **Solari A**., Fiaschi P., Zona G., Barbieri F., Florio T. "Isolation and characterization of putative stem cells from human meningiomas" BRAYN Conference, Genova, abs NO2 (2018).

5. **Solari A.**, Rogna L., Fiaschi P., Zona G., Barbieri F., Florio T. “Isolation and characterization of putative stem cells from human meningiomas”, poster presentation at CRUK Brain Tumour Conference, London, UK, 1-3 May, 2018.
6. **Solari A.**, Corsaro A., Bajetto A., Thellung S., Begani G., Villa V., Nizzari M, Pattarozzi A. Gatti M., Pagano A., Würth R., Daga A., Barbieri F., Florio T. “Silencing of cellular prion protein impairs the stem properties of human glioblastoma cancer stem cells”, poster presentation at BYNS 2017, Barcelona, Spain, 15-16 June, 2017.
7. Mattioli F., Marini V., Fucile C., **Solari A**., Daga A., Pattarozzi A., Baldassarri S., Zuccari G., Barbieri F, Caviglioli G., Florio T. “Subcutaneous slow-release metformin formulation grants high tissue drug levels and induce antitumoral effects. An in vivo feasibility pilot study”, 38° Congresso della Società Italiana di Farmacologia (Rimini, 25-28 ottobre) abs 2/23 (2017).

   2nd place at “Royal Society of Chemistry Twitter Poster Conference - Chemical Biology #RSCChemBio category ” with the poster 09 March 2018.
8. **Solari A.**, Corsaro A., Bajetto A., Thellung S., Begani G., Villa V., Nizzari M, Pattarozzi A. Gatti M., Pagano A., Würth R., Daga A., Barbieri F., Florio T. “Silencing of cellular prion protein impairs the stem properties of human glioblastoma cancer stem cells”, poster presentation at ENCODS 2017, Benidorm, Alicante, Spain, 4-6 May, 2017.

Prizes for scientific pictures

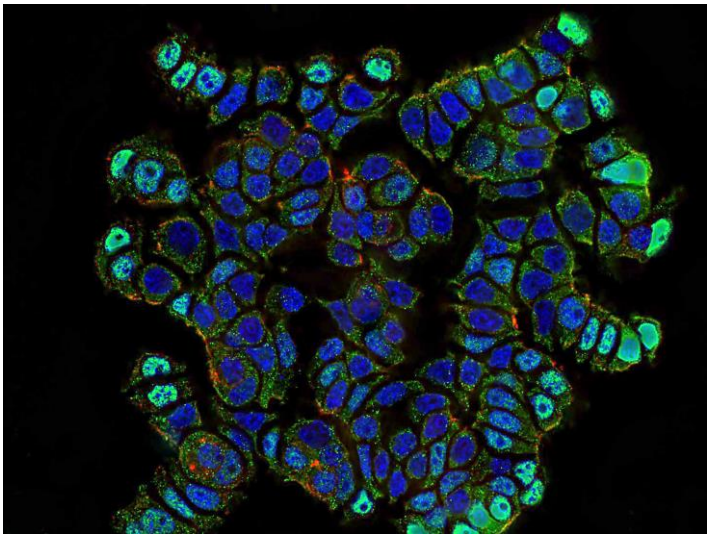
Potent Potential



3rd place in the DRVISION and Interstellate Neuroscience Travel Award Image Contest 2017 for SfN17 in Washington DC.

The image has been included into Interstellate Volume 2.

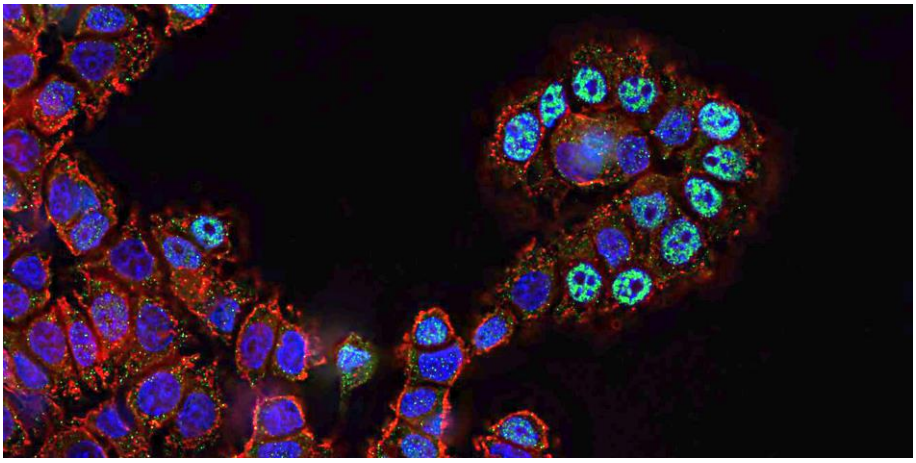
Pituitary Ballroom



Selected for Ibidi calendar 2019 (January)

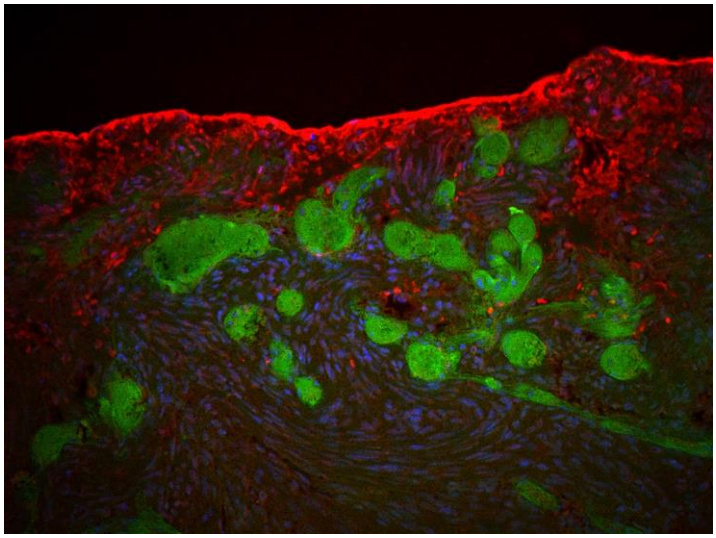
Fluorescence image of rat pituitary GH4 tumor cells treated with Octreotide for five days. The Somatostatin receptor 2 was specifically stained using an SSTR2 antibody (green), showing internalization of external SSTR2. The membrane was stained with phalloidin (red).

GH4 - SSTR2 & Phalloidin



Selected Image for the Contest "Arte o Scienza? - Immaginario scientifico" for SHARPER-La Notte Europea dei Ricercatori e Trieste Next, Trieste, 28-30 September 2018.

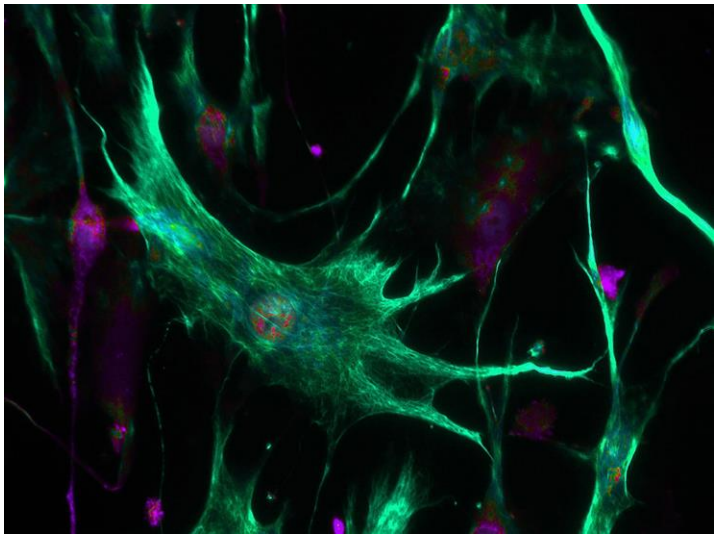
Meningioma Landscape



1st place at "II Barcelona Young Neuroscientists Symposium, NeuroArt Contest", 2018

Representative CD31 (green), CXCR4 (red) and nuclei (DAPI, blue) staining of a paraffin fixed meningioma section.

Ghost cells



Winner of People's Choice award NeuroArt Image Contest, MBF Bioscience, May 2018

Human primary cell cultures of meningioma. Cells were stained with anti-Vimentin (green) and DAPI (violet).

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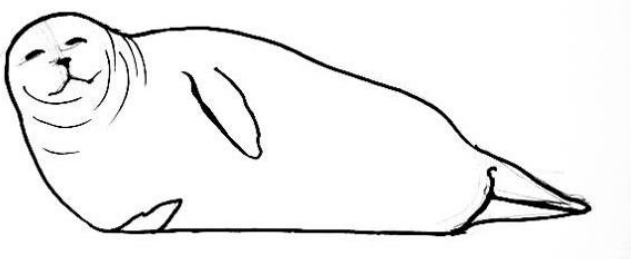

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